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Tesi

"Vascular dysfunction in a mouse model of Rett Syndrome and effects of Curcumin treatment"

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Riassunto

Mutazioni nel gene legato al cromosoma X, MeCP2 (Methyl CpG-binding protein) sono presenti in circa l'80% dei pazienti affetti da Sindrome di Rett, una delle più comuni cause di ritardo mentale nelle bambine e per cui ancora oggi non esistono efficaci trattamenti farmacologici. Un aspetto rilevante, ma ancora poco esplorato, nei pazienti con sindrome di Rett è la riduzione della circolazione periferica.

Per investigare la relazione tra la perdita di MeCP2 e questo aspetto clinico abbiamo utilizzato per gli studi funzionali, farmacologici e comportamentali, topi mancanti del gene MeCP2, maschi ($MeCP2^{y/-}$) e femmine ($MeCP2^{+/-}$). Gli studi funzionali condotti su branche dissezionate delle arteriole dell'albero mesenterico montate su microcannule di vetro in un miografo a pressione, hanno mostrato una drammatica riduzione della reattività vascolare endotelio dipendente nei topi $MeCP2^{+/-}$ rispetto ai controlli sani. Le arteriole preincubate con inibitori delle NOS o con acido ascorbico hanno rilevato una ridotta biodisponibilità di Ossido Nitrico (NO) e un aumento nelle specie reattive dell'ossigeno (ROS). Inoltre, l'animale Rett presenta bassi livelli di espressione sia dell'mRNA che del peptide eNOS nelle arteriole e alti livelli di stress ossidativo. Da un punto di vista comportamentale i topi knockout per MeCP2 mostrano comportamenti stereotipati e si osserva una riduzione del tempo di riposo.

Il trattamento cronico delle femmine $MeCP2^{+/-}$ con curcumina è in grado di ripristinare il normale fenotipo e di migliorare la sintomatologia comportamentale della Rett, diminuendo i caratteristici movimenti stereotipati e aumentando il tempo di riposo degli animali.

Questi dati indicano che la mancanza del gene MeCP2 modifica la circolazione periferica alterando la reattività vascolare, riducendo i livelli di espressione di eNOS e di NO. Inoltre, i nostri risultati supportano sia dal punto di vista funzionale/molecolare che comportamentale l'utilizzo della curcumina nella terapia dei pazienti affetti da sindrome di Rett.

Abstract

Mutations in the coding sequence of the X-linked gene MeCP2 (Methyl CpG–binding protein), are present in around 80% of patients with Rett Syndrome, a common cause of mental retardation in female and to date without any effective pharmacological treatment. A relevant, and so far unexplored feature of RTT patients is a marked reduction in peripheral circulation.

To investigate the relationship between loss of MeCP2 and this clinical aspect, we used a MeCP2 null mouse model, male ($MeCP2^{y/-}$) and female ($MeCP2^{+/-}$), for functional, pharmacological and behavioural studies. The functional studies performed on dissected branches of mesenteric arterial tree mounted on glass microcannule in a pressurized myograph, demonstrated a dramatic endothelial-dependent vascular reactivity impairment in $MeCP2^{+/-}$ compared to control littermate. The mesenteric arteriole preincubation with NOS inhibitors or ascorbic acid indicate a decrease Nitric Oxide (NO) availability and the increased presence of Reactive Oxygen Species (ROS). Consistently, the RTT mouse model exhibited a decreased expression in both mRNA and peptide eNOS in the arterioles and a higher systemic oxidative level. MeCP2 knockout mice show stereotyped movements and less resting time when compared to control littermates.

Chronic curcumin treatment of female $MeCP2^{+/-}$ mice was able to reverse this vascular phenotype and ameliorate the mouse RTT behavioural symptomatology by decreasing stereotyped movements and by increasing resting time.

These data indicate that in the absence of MeCP2 peripheral circulation is impaired by an altered vascular reactivity and decreased arteriolar eNOS expression and NO production. Further, they provide a physiological/molecular rational for the use of curcumin as a treatment to improve the health of RTT patients.

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INTRODUCTION

1.1Pervasive developmental disorders: an overview

Pervasive developmental disorders (PDD), also called autism spectrum disorders, comprise a complex and heterogeneous group of pathological conditions characterized by abnormalities of brain development and function, appearing within the first three years of life. Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DMS-IV) identify as PDD five pathology: autism, Rett syndrome, childhood disintegrative disorder, Asperger disorder and PDD not otherwise specified under the spectrum of PDD (PDD-NOS) (American Psychiatric Association., 2000) (Figure 1).

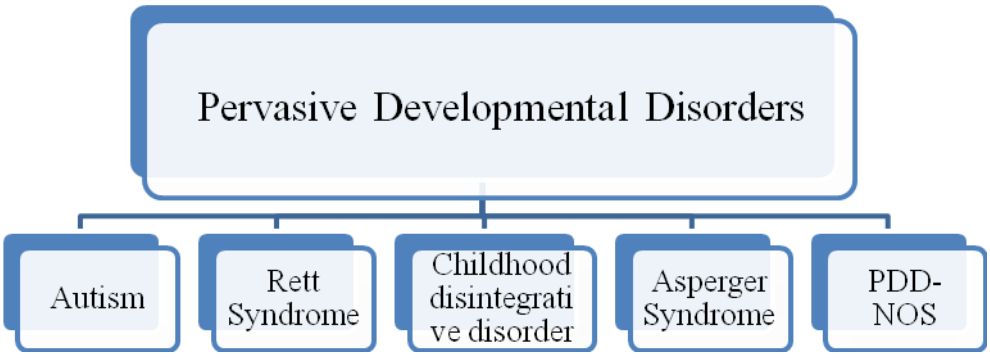


Figure 1: Pervasive Developmental Disorders (PDD). Current Classification in DMS-IV.

Although the detailed causal mechanisms are not known, autism is likely to have multiple etiologies including genetic factors. Children with PDD share the following characteristics:

i) impairments in social interaction;

ii) inability to imaginative activity;

iii) problems in verbal and nonverbal communication skills;

iv) stereotyped behaviors;

v) cognitive deficits;

vi) limited number of interests and activities.

Children affected by PDD have difficult in talking, playing with other children, and relating to others, including their family.

The incidence of these disorders has risen dramatically over the past two decades mainly because of the use of broader diagnostic criteria and the increased attention of the medical community (Levy et al, 2009) and is estimated 60-70/10000 children. PDD is 4/5 times more frequently in boys, with the exception of Rett syndrome, which is found mainly in girls. Great interest exists in the elucidation of the causes and pathogenesis of autism. Although autism is recognized to be the common endpoint of neurological dysfunction of varying etiologies, common disease mechanisms may underlie the phenotypes shared by RTT and autism, and advances in understanding of RTT may also shed some light into the pathogenesis of autism.

1.2 Rett Syndrome

Rett syndrome (RTT) is a neurological disorder that predominantly affects girls (Hagberg et al, 1985; Hagberg & Hagberg, 1997). It represents the second most common cause of mental retardation in females with an incidence of 1:10000 (Percy, 2002). The disease has been described for the first time by the Austrian neurologist Andreas Rett in 1966, but the scientist community recognized the pathology only 17 years later when the Swedish neurologist Dr. B. Hagberg described 35 new cases of RTT (Hagberg et al, 1983).

In 1954, the Viennese pediatrician Dr. Andreas Rett observed two girls in his waiting room displaying the same repetitive hand-washing motions. Looking at their clinical charts, he realized they shared similar clinical and developmental histories. Dr. Rett soon recognized that six other girls in his practice had similar behaviors, and reasoned that these girls must have the same disorder, which at that time was new to the medical field. In fact, their distinctive behavioral patterns indicated to him that this new condition was not a simple mental retardation syndrome, but rather a complex condition that affected several facets of neural function. Unfortunately, during the 1960's in Europe the medical community was hesitant to recognize "new" conditions in the absence of a metabolic abnormality. This led Dr. Rett to examine several physiological parameters in his affected girls, where he found elevated ammonia content in blood. In 1966, Dr. Rett published his observations in a leading German medical journal, where he described the condition as one of cerebral atrophy and hyperammonia in girls, characterized by autistic behavior, dementia, and apraxia of gait (Rett, 1966). The condition stayed mostly unrecognized in the English language literature until 1983, when Bengt Hagberg described 35 patients, all girls from three countries (France, Portugal and

Sweden), with a uniform and striking, progressive encephalopathy. At this point, he revisited Rett's unusual mental retardation syndrome and suggested the condition bear his name (Hagberg et al, 1983). In 1991, Bruck and colleagues described a set of monozygotic female twins with Rett syndrome (Bruck et al, 1991).

The genetic cause of RTT remained evasive until quite recently, largely because the inheritance pattern was chiefly sporadic. In 1999, Amir and colleagues (Amir et al, 1999) discovered that mutations in the gene encoding Methyl-CpG-binding protein 2 (MeCP2) are associated both with rare familial cases of RTT as well as with the more common sporadic occurrences of typical RTT. This mutation is responsible of 95% of RTT cases (Amir et al, 1999; Van den Veyver & Zoghbi, 2000; Wan et al, 1999; Xiang et al, 2000). Further, mice lacking MeCP2 display Rett-like symptoms, and this phenotype can be reversed with the reintroduction of MeCP2 (Guy et al, 2007).

1.2.1 Clinical features of Rett Syndrome

Girls with RTT born after an apparently quiet pregnancy. They are normal at the birth and achieve expected developmental milestones until 6-18 months of age. Nevertheless, some studies revealed subtle behavioral abnormalities soon after birth. There is a wide variability in the clinical presentation, classic Rett syndrome follows a well recognized pattern. Not all symptoms compare at the beginning of the disease but can be distinguish four different stages (Figure 2).

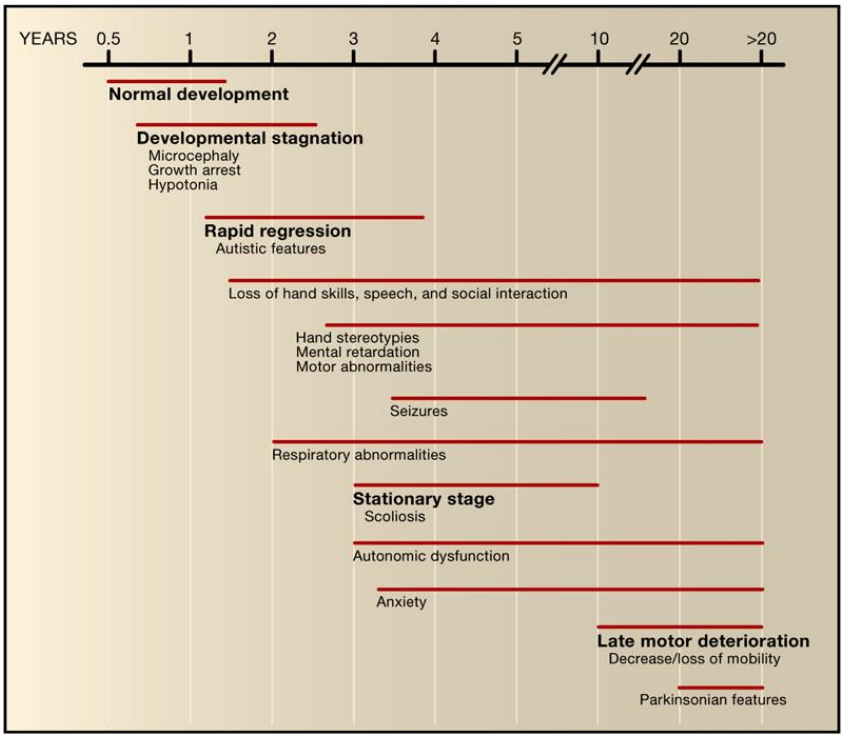


Figure 2: Onset and Progression of RTT Clinical Phenotypes.

In the development of the disorder, the first indicator of neurological involvement is deceleration of head growth, leading to microcephaly by the second year of life. This decreased head growth is due to decreased neuronal growth. The acquired microcephaly is accompanied by general growth retardation, weight loss, and a weak posture brought on by muscle hypotonia.

Subsequently there is a period of rapid regression and patients lose purposeful use of their hands and instead develop stereotypic hand wringing or washing movements, and in some cases clapping, flapping, and mouthing of the hands. Social withdrawal and loss of language become apparent in addition to irritability and self-abusive behavior. Other autistic features also manifest, including expressionless face, hypersensitivity to sound, lack of eye-to-eye contact, indifference to the surrounding environment, and unresponsiveness to social cues (Nomura, 2005). The onset of mental deterioration is accompanied by loss of

motor coordination and the development of ataxia and gait apraxia. The earliest autonomic perturbation is hyperventilation during wakefulness. Most girls with RTT suffer additional breathing anomalies, including breath-holding, aerophagia, forced expulsion of air and saliva, and apnea. One of the most arduous features of RTT is the occurrence of seizures, which range from easily controlled to intractable epilepsy, with the most common types being partial complex and tonic-clonic seizures (Jian et al, 2006). The seizures tend to decrease in severity after the teenage years and into adulthood, presenting minor problems after the age of forty.

The third stage of the pathology is characterized by an amelioration of the social component of the autistic-like behavior occurs sometime between 5 to 10 years of age. Behavioral abnormalities during this post regression phase include teeth grinding, night laughing or crying, screaming fits, low mood, and anxiety episodes elicited by distressful external events (Mount et al, 2001). Patients suffer devastating motor deterioration, generalized rigidity, dystonia, and worsening of scoliosis. Most girls with RTT lose mobility, and are often wheelchair-bound during the teenage years. Sleep problems are common in Rett syndrome (in over 80% of cases), specifically has been shown disrupted sleep patterns at night and an increase in total and daytime sleep (Young et al, 2007). Additional autonomic abnormalities include hypotrophic, severe constipation, oropharyngeal dysfunction, and cardiac abnormalities, including tachycardia, prolonged corrected QT intervals, sinus bradycardia and cold blue feet. Despite their excellent appetites, individuals with RTT commonly present eating problems (Reilly & Cass, 2001) and not have a correct intake of calories in the diet, especially during pre-school and early school years. This can be caused by

problems in coordinating movements of the mouth and throat, muscle spasms and involuntary movements, the expenditure of large amounts of energy when breathing and the inability of the intestine to absorb nutrients. They have difficult to co-ordinate breathing and swallowing. Patients continue to lose weight and many suffer from osteopenia, scoliosis, and rigidity as they age. As patients get older they often develop Parkinsonian features (Hagberg, 2005; Roze et al, 2007). The condition reaches a plateau and some patients survive up to the sixth or seventh decade of life in a severely debilitated physical condition.

1.2.2 The autonomic nervous system

Certain physical functions including the regulation of heart rate, blood pressure, peripheral blood circulation, respiration and digestion are governed by the autonomic nervous system. In Rett syndrome there are varying degrees of dysfunction in the control of the central autonomic nervous system.

Abnormal breathing patterns affecting pulmonary and cardiovascular function are characteristic of Rett syndrome. They can be divided into three categories: forceful breathing, abnormally shallow breathing and apneustic breathing (a series of slow deep inspirations). Valsalva breathing (attempting to forcibly exhale while the epiglottis is closed) is a common, complication to the breathing abnormalities characteristic of Rett syndrome, and affects the autonomic nerve system and brain stem functions. The consequences for the individual of Valsalva breathing depend on the category of breathing abnormality he or she manifests(Julu et al, 2001).

Impaired balance of central autonomic control may also result in cold, bluish, clammy feet due to peripheral vasomotor disturbances. Hydrotherapy, and

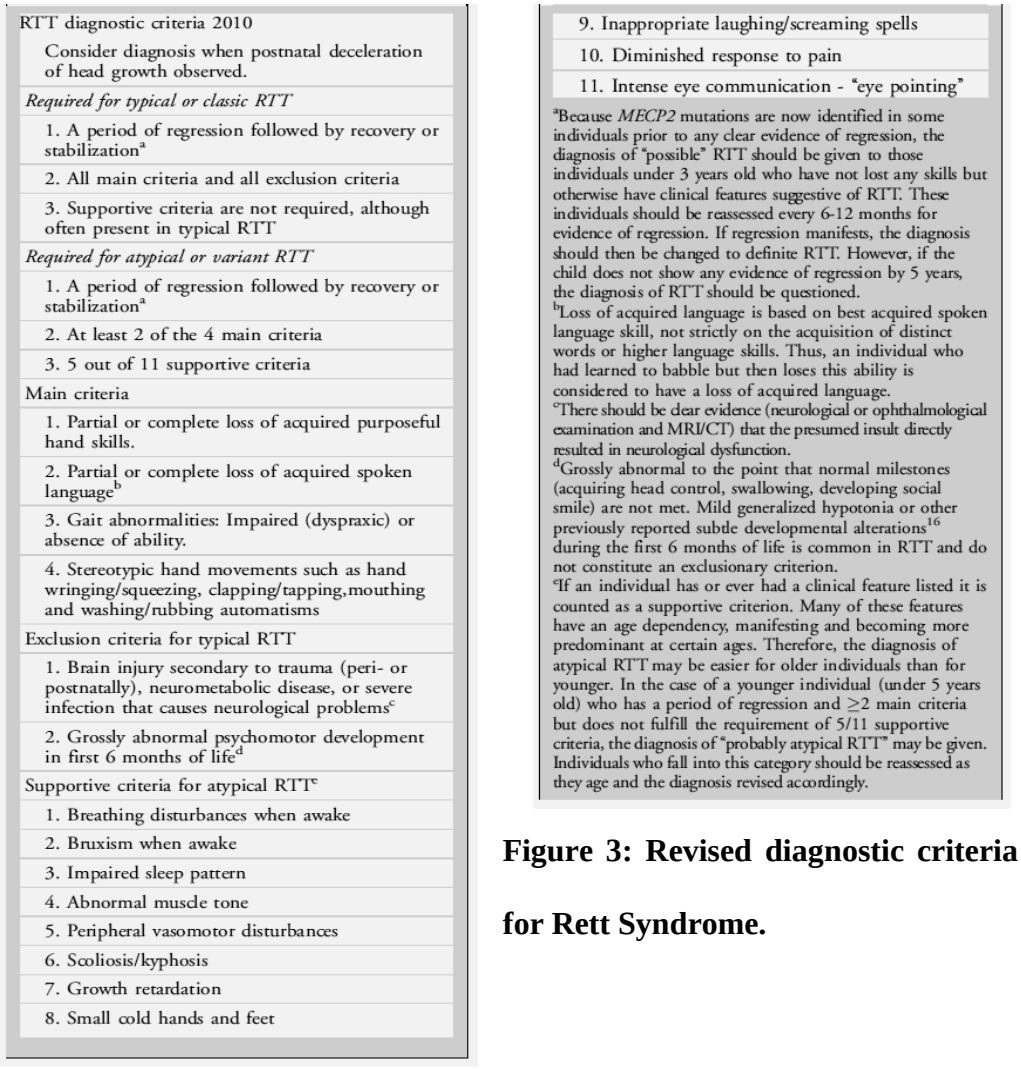
physiotherapy to the extremities is often used to regain proper circulation and helps to keep their extremities limber (Stearns et al, 2007).

The motility of the gastrointestinal tract (peristalsis) shifts the food along the tract and is controlled and coordinated by the autonomic nervous system. It is common that these movements are impaired and that the passage of food through the tract is unusually slow, leading to a number of symptoms of varying degrees of severity. The girls have problems swallowing and may swallow air resulting in an extended, painful stomach, and vomiting. Painful inflammation in the lower esophagus (esophagitis) can occur from an early age if the lower esophageal sphincter leaks. Severe constipation is very common.

1.2.3 Clinical criteria for typical Rett Syndrome

In 1994 Hagberg and Skjeldal suggested a model of inclusion and exclusion criteria for the diagnosis of RTT that relaxed the international criteria originally drawn up in Vienna in September 1984 (Hagberg & Skjeldal, 1994). In 2002 the same authors updated the previous diagnostic criteria (Hagberg et al, 2002). In 2010 researchers reviewed 2002 diagnostic criteria (Figure 3) to clarify and simplify the diagnosis of typical, or classic, RTT. They emphasized that it remains a clinical diagnosis, since not all RTT patients have MECP2 mutations and not all patients with MeCP2 mutations have Rett syndrome. They limited the necessary criteria to the presence of a period of clear developmental regression plus four main criteria that are absolutely required for the diagnosis of typical RTT: loss of purposeful hand skills, loss of spoken language, gait abnormalities, and stereotypic hand movements. After the period of regression, a stage of stabilization and potentially even improvement ensues, with some individuals

partially regaining skills. This potential for some skill recovery emphasizes the importance of the acquisition of a careful history to determine the presence of regression. Furthermore they eliminated postnatal deceleration in head growth from the necessary criteria because this feature in not found in all individuals with typical RTT. However, because it is a clinical feature that can alert a clinician to the potential diagnosis and it is a distinctive feature in the disorder, this has been included as a preamble to the criteria as a feature that should raise suspicion for the diagnosis. In these new criteria, history of regression and all of the necessary and exclusion criteria must be met to make the diagnosis of typical RTT, without exception (Neul et al, 2010).



RTT diagnostic criteria 2010
Consider diagnosis when postnatal deceleration of head growth observed.
Required for typical or classic RTT
1. A period of regression followed by recovery or stabilization[a]
2. All main criteria and all exclusion criteria
3. Supportive criteria are not required, although often present in typical RTT
Required for atypical or variant RTT
1. A period of regression followed by recovery or stabilization[a]
2. At least 2 of the 4 main criteria
3. 5 out of 11 supportive criteria
Main criteria
1. Partial or complete loss of acquired purposeful hand skills.
2. Partial or complete loss of acquired spoken language[b]
3. Gait abnormalities: Impaired (dyspraxic) or absence of ability.
4. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping,mouthing and washing/rubbing automatisms
Exclusion criteria for typical RTT
1. Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems[c]
2. Grossly abnormal psychomotor development in first 6 months of life[d]
Supportive criteria for atypical RTT[e]
1. Breathing disturbances when awake
2. Bruxism when awake
3. Impaired sleep pattern
4. Abnormal muscle tone
5. Peripheral vasomotor disturbances
6. Scoliosis/kyphosis
7. Growth retardation
8. Small cold hands and feet
9. Inappropriate laughing/screaming spells
10. Diminished response to pain
11. Intense eye communication - "eye pointing"

[a]Because *MECP2* mutations are now identified in some individuals prior to any clear evidence of regression, the diagnosis of "possible" RTT should be given to those individuals under 3 years old who have not lost any skills but otherwise have clinical features suggestive of RTT. These individuals should be reassessed every 6-12 months for evidence of regression. If regression manifests, the diagnosis should then be changed to definite RTT. However, if the child does not show any evidence of regression by 5 years, the diagnosis of RTT should be questioned.
[b]Loss of acquired language is based on best acquired spoken language skill, not strictly on the acquisition of distinct words or higher language skills. Thus, an individual who had learned to babble but then loses this ability is considered to have a loss of acquired language.
[c]There should be clear evidence (neurological or ophthalmological examination and MRI/CT) that the presumed insult directly resulted in neurological dysfunction.
[d]Grossly abnormal to the point that normal milestones (acquiring head control, swallowing, developing social smile) are not met. Mild generalized hypotonia or other previously reported subtle developmental alterations[16] during the first 6 months of life is common in RTT and do not constitute an exclusionary criterion.
[e]If an individual has or ever had a clinical feature listed it is counted as a supportive criterion. Many of these features have an age dependency, manifesting and becoming more predominant at certain ages. Therefore, the diagnosis of atypical RTT may be easier for older individuals than for younger. In the case of a younger individual (under 5 years old) who has a period of regression and ≥2 main criteria but does not fulfill the requirement of 5/11 supportive criteria, the diagnosis of "probably atypical RTT" may be given. Individuals who fall into this category should be reassessed as they age and the diagnosis revised accordingly.

Figure 3: Revised diagnostic criteria for Rett Syndrome.

1.2.4 Atypical forms of Rett Syndrome

In addition to the classic form of RTT, five distinct categories of variants have been delineated on the bases of clinical criteria (Hagberg et al, 2002; Trevathan & Naidu, 1988). These variants show some, but not all diagnostic features of RTT and can be milder or more severe. They include:

i) the **preserved speech variant** (PSV), is the best characterized, has well-defined clinical features, and mutations in MECP2 have been found in the majority of cases; in this variant girls recover the ability to speak in single word or third person phrases and display an improvements of purposeful hand movements at stage III of disease progression (Zappella et al, 2001);

ii) the **early seizures variant (Hanefeld Variant)** , in which the normal perinatal period is soon followed by the appearance of seizures preceding the regression period (Hanefeld, 1985);

iii) the **"forme fruste"** with a milder, incomplete and protracted clinical course (Hagberg & Rasmussen, 1986; Hagberg & Witt-Engerstrom, 1986);

iv) the **congenital variant**, where patients show RTT features straight from birth (Rolando, 1985);

v) the **late regression variant**, which is characterized by normal head circumference and gradual loss of acquired speech and fine motor skills in late childhood (Gillberg, 1989).

Furthermore, it has been described a "highly functioning PSV" associated with acquisition of more complex language function including the use of first person phrases. In this variant, girls acquire a better control of their hands and they are

able to draw figures and write simple words (Zappella et al, 2003). The degree of mental retardation is milder then in PSV with the I.Q that can be as high as 50.

Recent works have found mutations in different loci associated with congenital and Hanefeld variants, with mutations in FOXG1 found in congenital variant cases (Ariani et al, 2008) and mutations in CDKL5 found in early seizure variant cases (Bahi-Buisson et al, 2008).

1.2.5 Genetic origin of Rett Syndrome

Early reports postulated an X-linked dominant mode of inheritance with fateful consequences in homozygous males. Using information from rare familial cases, exclusion mapping identified the Xq28 candidate region, and subsequent screening of candidate genes in RTT patients revealed mutations in MECP2 (Amir et al, 1999). Mutations in MECP2 are found in more than 95% of classic RTT cases, most arise de novo in the paternal germ line and often involve a C to T transition at CpG dinucleotides. The spectrum of mutation types includes missense, nonsense, and frameshift mutations, with over 300 unique pathogenic nucleotide changes described (Christodoulou & Weaving, 2003), as well as deletions encompassing whole exons (Archer et al, 2006; Pan et al, 2006; Ravn et al, 2005). Eight missense and nonsense mutations account for 70% of all mutations, while small C-terminal deletions account for another 10%, and complex rearrangements constitute 6%.

1.2.6 Effect of XCI

In each body cell (somatic cell) of the developing baby girl, one of the X chromosomes becomes very shortened and condensed so that most of its genes are not able to be 'read' by the cells.

This system of inactivation in the body cells known as X-chromosome inactivation (XCI) is totally normal in female and is usually random so that women's bodies have a mixture of cells in regard to the inactivated X chromosome, although certain genes on the silenced chromosome may still be expressed. Some cells will have the X chromosome switched off that came from their mother (an inactive maternal X chromosome); other cells will have the paternal X chromosome inactivated. The relative proportion of cells with an active maternal or paternal X chromosome varies from female to female (even between identical twins) because the process is usually random. In rare situations, females can show nonrandom or skewed X-inactivation with the preferential inactivation of one X-chromosome in most or all of the cells in the body. In these cases, females may be protected from expression of X-linked diseases if the X-chromosome with the abnormal gene is inactivated. XCI only occurs in the somatic cells, since both X chromosomes need to be active in the egg cells for their normal development.

Of course this process occurs also in Rett patients and can produce a pattern favoring the expression of the X chromosome containing the normal MECP2 gene, or mutated MECP2 gene. Asymptomatic carriers of RTT can have highly skewed inactivation patterns of XCI favoring the normal MECP2 allele. Sisters with identical MECP2 mutations can have extremely discordant phenotypes as a result of XCI skewing. One sister displayed classic Rett Syndrome while the other

was a “highly functioning” preserved speech variant as a result of favourable XCI skewing (Scala et al, 2007). XCI patterns may interact with mutation type, so that Rett patients with severe early truncating mutation present a milder phenotype than would be expected. The process of XCI also occurs in Mecp2-deficient female mice models (Young & Zoghbi, 2004). In Mecp2-heterozygotes, XCI patterns generally favour the wild-type allele which may in part explain the mild phenotype observed (Young & Zoghbi, 2004).

1.2.7 Genotype/Phenotype correlations

Several studies have addressed the relationship between genotype and phenotype directly using scoring systems and statistical methods to compare the severity of individual clinical features, with different types of MeCP2 mutations (missense mutations vs. nonsense mutations, early truncations vs. late truncating mutations, MBD mutations vs. TRD mutations). They evidenced that RTT patients present a large phenotypic variability associated with different MeCP2 mutations. Recent genotype-phenotype studies showed that severity of RTT phenotype depends on the type of the mutation, the genetic background and the X-chromosome inactivation (XCI) balance.

Patients carrying mutations that truncate the protein in the C-terminal domain (late truncating mutations) present milder phenotype and are less typical of classical Rett Syndrome than those carrying missense or early truncating mutations. Jian reported in 2005 that R270X mutation (X representing here a stop codon) is associated with elevated mortality; whereas Wan showed that girls carrying the same mutations could sometimes present different phenotypes. This

observation is consistent with an important role for the XCI balance (Jian et al, 2005; Wan et al, 1999).

1.2.8 MeCP2 Mutations In Boys

At the beginning, RTT was considered to be an X-linked dominant disorder girls with Rett syndrome would be heterozygous for the defective gene (one normal X-chromosome and one X-chromosome with the defective gene) and males, who only have one X-chromosome, would not survive the gene defect.

In 1999, Jan and colleagues identified males with Rett syndrome (Jan et al, 1999). Interestingly, males with MeCP2 mutations can be classified in three ways:

1) MeCP2 mutations normally observed in girls with Rett syndrome result in a more severe clinical phenotype in hemizygous males (Hoffbuhr et al, 2001; Villard et al, 2000; Wan et al, 1999)
2) Classical Rett syndrome and nonfatal neurodevelopmental disorder with similarities to Rett syndrome have been reported in an XXY Klinefelter's male with a T158M MeCP2 mutation (Hoffbuhr et al, 2001) and a boy showing somatic mosaicism for 2 bp deletion in the MeCP2 gene (Clayton-Smith et al, 2000)
3) A subset of novel MeCP2 mutations (A140V, Q406X, G428S, E137G, R167W, P399L, R453Q), not identified in girls with Rett syndrome, has been found in several familial cases and five sporadic cases of nonspecific X-linked mental retardation in boys (Couvert et al, 2001; Imessaoudene et al, 2001; Meloni et al, 2000; Orrico et al, 2000).

1.3 MECP2 GENE: Structure and Function

The MECP2 gene is located in Xq28 between the IRAK and RCP genes. The MeCP2 protein is a chromatin-associated protein identified and purified for the first time in 1992 by Dr Adrian Bird on basis of its capacity to bind methylated DNA (Lewis et al, 1992) (Figure 4). MeCP2 is the "founding member" of the methylated DNA binding protein (MBP) family. In order it is also the first MBP found to interact with HDAC-containing complexes, linking two epigenetic repression mechanism: DNA methylation and histone deacetylation (Nan et al, 1998). It is required for maturation of neurons and is developmentally regulated (Swanberg et al, 2009).

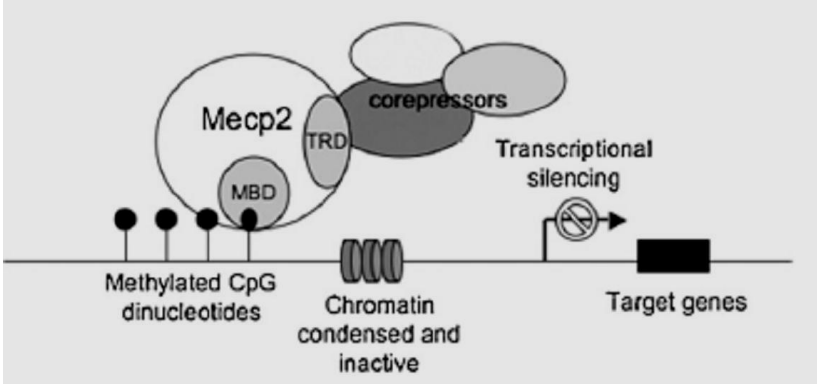


Figure 4: Transcriptional repression by MeCP2.

The protein is present in all vertebrates, including the sea lamprey, a primitive jawless vertebrate, but no MeCP2 ortholog has been detected in invertebrate animals or in plants. Among mammals the MeCP2 protein is highly conserved. Sequences from human and mouse, for example, which diverged from a common ancestor ~75 million years ago, are 95% identical at the amino acid level. Divergences between mammalian MeCP2 and amphibian or fish MeCP2

are more extensive (33% amino acid identity between human and zebrafish), but conserved domains are present.

MECP2 consists of four exons that code for two different isoforms of the protein produced by alternative splicing of a short segment at the extreme N-terminus of the protein (Figure 5).

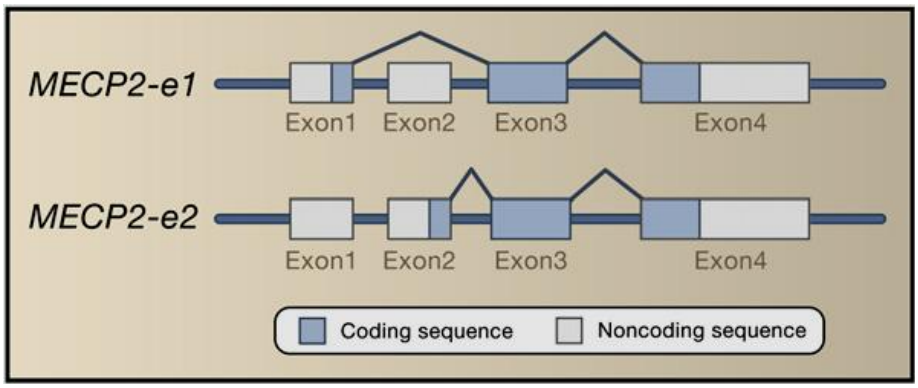


Figure 5: Alternative splicing forms of the MECP2: MECP2-e1 and MECP2-e2.

The MeCP2-e2 isoform was the first identified variant of MeCP2 and therefore the best characterized, but the MeCP2-e1 isoform is more abundant in the brain of both mouse and human (Mnatzakanian et al, 2004).

The MECP2-e1 isoform contains 24 amino acids encoded by exon 1 and lacks the 9 amino acids encoded by exon 2, whereas the start site for the MECP2-e2 isoform is in exon 2 (Dragich et al, 2007; Kriaucionis & Bird, 2004; Mnatzakanian et al, 2004). The two splice variants differ in translation efficiency and are expressed at different relative amounts in different tissues. MECP2-e1 is more abundant in the brain, thymus and lung and during neuronal differentiation. However, recent studies show that both of these isoforms co-localize to heterochromatic regions in murine fibroblastic cells (Kumar et al, 2008). These

two MeCP2 isoforms present differences in structure and distribution, but not in function. MeCP2 protein levels are low during embryogenesis and increase progressively during the postnatal period of neuronal maturation (Balmer et al, 2003; Cohen et al, 2003; Kishi & Macklis, 2004; Mullaney et al, 2004; Shahbazian et al, 2002b). Both MeCP2 isoforms are nuclear and co-localize with methylated heterochromatic foci in mouse cells. A recent report suggests that MeCP2 translocates to the nucleus upon neuronal differentiation (Miyake & Nagai, 2007).

Since MeCP2 is expressed in mature neurons and its levels increase during postnatal development, MeCP2 may play a role in modulating the activity or plasticity of mature neurons. Consistent with this, MeCP2 mutations do not seem to affect the proliferation or differentiation of neuronal precursors. Although the mechanisms that regulate the complex MeCP2 expression patterns are unknown. Recent studies identified the core promoter and several cis-regulatory elements that drive MeCP2 expression (Liu & Francke, 2006). These regulatory sequences may dictate the spatial and temporal patterns of MeCP2 expression.

MeCP2 is composed of three domains: the methyl-binding domain (MBD), the transcriptional repression domain (TRD), and a C-terminal domain, in addition to two nuclear localization signals (NLS) (Figure 6).

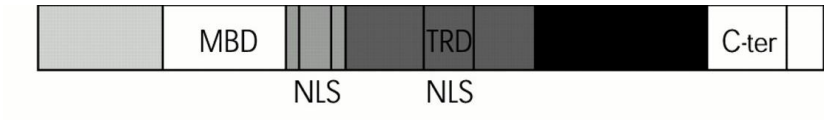


Figure 6: MeCP2 function domains. MBD methyl-binding domain; TRD transcriptional repression domain; C-ter C-terminal domain and NLS nuclear localization signals.

The MBD, an 85 amino acids domain is located on exons 3 and 4 (from amino acid 78 to 162 in MeCP2), and specifically binds to methylated CpG dinucleotides, with preference for CpG sequences with adjacent A/T-rich motifs (Klose et al, 2005). MBD also binds to unmethylated four-way DNA junctions with a similar affinity (Galvao & Thomas, 2005), implicating a role for the MeCP2 MBD in higher-order chromatin interactions. The TRD is a 104 amino acids domain, situated on exon 4 (from amino acid 207 to 310), important in generating a physical association with the transcriptional corepressor Sin3a, which recruits the histone deacetylases HDAC1 and HDAC2. These histone deacetylases remove acetyl groups from histones, resulting in a compact chromatin structure that represses local gene expression (Jones et al, 1998; Nan et al, 1997). The C-terminal region of MeCP2 is not yet well characterized, it is clearly essential for protein function as evidenced by the numerous RTT-causing mutations that involve deletion of this domain, and the fact that a mouse model lacking the MeCP2 C-terminus reproduces many RTT phenotypes (Shahbazian et al, 2002a). The C-terminal region has been described as containing a WW binding domain important for MeCP2 interactions with splicing factors (Buschdorf & Stratling, 2004). WW domains are characterized by the presence

of 2 tryptophan residues (W) that are separated by 20-22 amino acids and that recognize proline residues of interacting ligands.

The function of MeCP2 as a transcriptional repressor was first suggested on the basis of in vitro experiments in which MeCP2 specifically inhibited transcription from methylated promoters (Nan et al, 2007). When MeCP2 binds to methylated CpG dinucleotides of target genes via its MBD, its TRD recruits the corepressor Sin3A and HDAC 1 and HDAC 2 (Jones et al, 1998; Nan et al, 1998)(Figure 7).

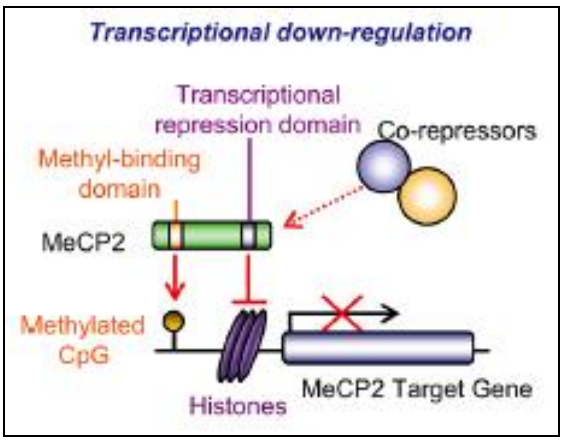


Figure 7: MeCP2 as a transcriptional repressor. MeCP2 binds to methylated CpG upstream of the transcriptional start site of a MeCP2 target gene.

The transcriptional repressor activity of MeCP2 involves compaction of chromatin by promoting nucleosome clustering, either through recruitment of HDAC and histone deacetylation or through direct interaction between its C-terminal domain and chromatin (Nikitina et al, 2007). Additional MeCP2-interacting proteins include the catalytic component of the SWI/SNF chromatin-remodeling complex Brahma (at least in NIH 3T3 cells), the DNA methyltransferase DNMT1, the histone methyltransferase Suv39H1, the

transcription factors TFIIB and PU.1, the corepressors c-Ski and N-CoR, LANA, and the SWI2/SNF2 DNA helicase/ATPase responsible for a-thalassemia/ mental retardation syndrome X-linked (ATRX) (Harikrishnan et al, 2005; Kaludov & Wolffe, 2000; Kimura & Shiota, 2003; Nan et al, 2007).

In 2005, has been demonstrated that MeCP2 in addition to its role as a global repressor, acts as a splicing regulator (Young et al, 2005). The authors identified the RNA-binding protein Y box-binding protein 1 (YB1), a principal component of messenger ribonucleoprotein particles that controls multiple step of mRNA processing, as a MeCP2 binding partner. The functional significance of this interaction was investigated by determining whether the MeCP2-YB1 complex affects mRNA processing and splice-site selection. It has been shown that in MeCP2-deficient neurons, the splicing is altered, and aberrantly spliced transcripts can be produced (Young et al, 2005).

Given that MeCP2 interacts with other proteins, chromatin, DNA, and RNA, it is clearly a multifunctional protein, with roles in chromatin remodeling and RNA splicing.

In contrast to the data showing that MeCP2 is only a transcription inhibitor, in 2008, Chahrour and colleagues carried out a study to examine gene expression patterns in the hypothalamus of MeCP2-null and transgenic mice. Surprisingly, authors found that the majority of genes displaying altered expression were up-regulated rather than down-regulated by MeCP2 (Figure 8), and that, in these neurons, MeCP2 protein was directly associate with the transcriptional activator CREB1 (Chahrour et al, 2008). An increased level of CREB1 induces miR132 microRNA and represses MeCP2 translation suggesting a negative regulatory loop between MeCP2 and CREB1 (Klein et al, 2007). These results confirm

another study, in which, by using ChIP-on-chip analysis in SH-SY5Y cells, MeCP2 has been found associated more frequently with promoters that are also associated with RNA polymerase II (Yasui et al, 2007). All these data suggest that MeCP2 would be a "transcriptional modulator" rather than a repressor.

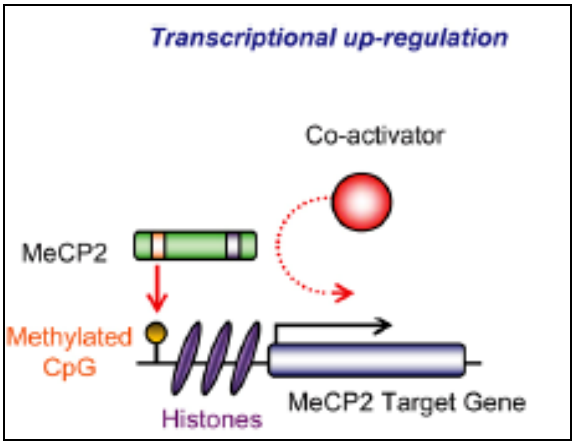


Figure 8: MeCP2 as a transcriptional activator. MeCP2 recruits a transcriptional coactivator to cause the transcriptional upregulation of a target gene.

1.3.1 Tissue expression of MeCP2

Several studies, carried out in rodents (Cassel et al, 2004; Mullaney et al, 2004; Shahbazian et al, 2002b) monkeys (Akbarian et al, 2001) and human (Shahbazian et al, 2002b), were aimed to analyze the expression profile of MeCP2. From these studies appears that MeCP2 is expressed in many tissues. Analysis on mice tissue samples revealed that the MeCP2 protein is not abundant in liver, stomach and small intestine, moderately expressed in kidney and heart and highly abundant in brain, lung and spleen (Shahbazian et al, 2002b). Within the cerebral tissue, MeCP2 is not abundant in astrocytes

(Maezawa et al, 2009) and immature neurons, but is preferentially expressed in mature neurons. The timing of its appearance correlated with the ontogeny of central nervous system. MeCP2 expression followed a similar time course in both mouse and human, with its early expression in the spinal cord and brainstem, and late expression in the hippocampus and cerebral cortex (Figure 9).

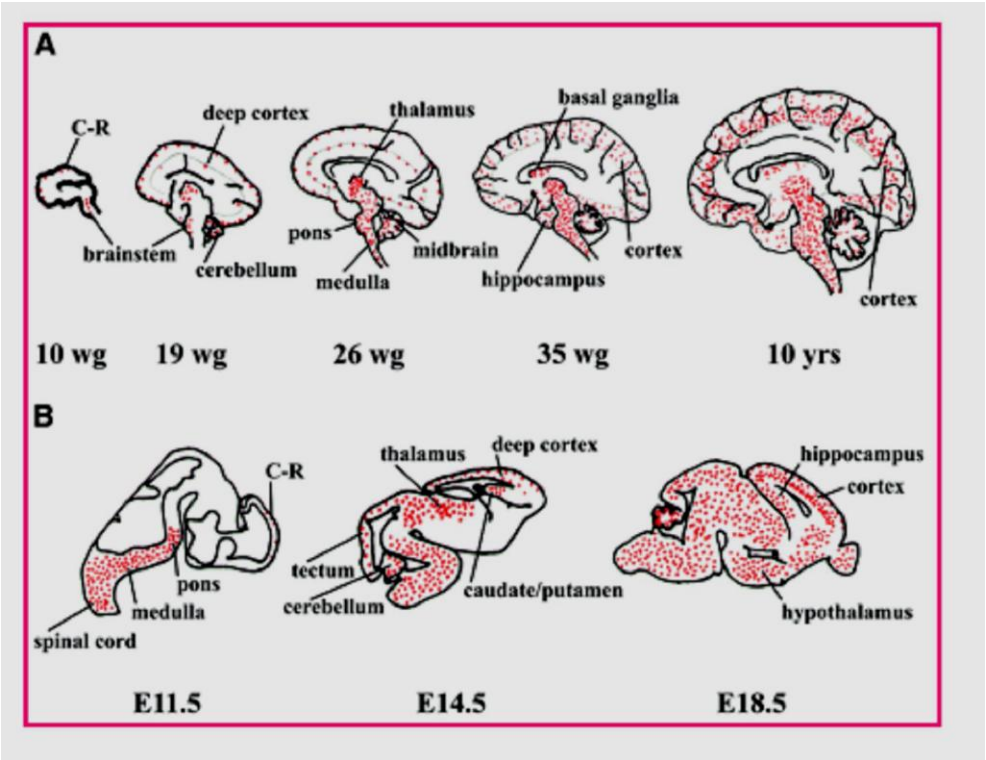


Figure 9: Schematic representation of the spatial and temporal distribution of MeCP2 during human (A) and mouse (B) development. Wg, weeks of gestation.

Direct quantification in adult mouse brain has estimated ~16 million molecules of MeCP2 per nucleus in neurons with almost an order of magnitude less in glial cells and 30-fold less in liver cells (Skene et al, 2010). The neuronal MeCP2 level is relatively low at birth but, in the mouse, increases greatly during the first

3 weeks of life before reaching a plateau (Kishi & Macklis, 2004; Skene et al, 2010). Because neurogenesis is largely complete before birth, the increase is due to up-regulation of MeCP2 expression within a constant number of neurons. These neurons are, however, developmentally active, undergoing synaptogenesis at this time. MeCP2 expression varies between neuronal populations from different regions and different structures within the central nervous system (LaSalle et al, 2001). MeCP2 expression is regulated in a developmental stage and cell type specific manner. Few things are known about molecular mechanisms implicated in this regulation. It was shown that the MeCP2 gene contains multiple transcription starting sites embedded in a region that is GC rich and contains CpG islands. In this study has been showed that the mouse MeCP2 promoter does not contain any canonical boxes like TATA or CAAT. They also identified a promoter region (-677/+56) that is responsible for the expression of MeCP2 in neuronal cells. In this region, there is a positive regulatory element of 19 bp (-64 to +46) that controls the major activity of the promoter region (Adachi et al, 2005).

Anatomically, MeCP2 deficiency causes reduced brain size. This may be due to size decreases in major brain regions such as the frontal and temporal lobes, caudate nucleus, thalamus, midbrain, and cerebellum, all of which have been documented in RTT patients (Armstrong et al, 2001; Reiss et al, 1993; Saywell et al, 2006; Subramaniam et al, 1997). At the cellular level, the neuronal soma is smaller in the absence of MeCP2, and cells are more densely packed, with decreased dendritic complexity (Armstrong, 2005; Chen et al, 2001; Kaufmann & Moser, 2000; Taneja et al, 2009). Importantly, degeneration and atrophy are not observed, establishing the notion that RTT is a postnatal developmental disorder,

rather than a neurodegenerative disorder (Armstrong, 2005; Jellinger et al, 1988; Jellinger & Seitelberger, 1986).

More specific abnormalities have been observed at the synapses. Postmortem brain samples from RTT patients or MeCP2-deficient mice present postsynaptic morphological defects such as reduced dendritic branching, reduced dendritic spine density, and defects in spine morphology (Armstrong et al, 1995; Armstrong et al, 2001; Belichenko et al, 1994; Chapleau et al, 2009; Fukuda et al, 2005; Kishi & Macklis, 2004; Schule et al, 2008; Smrt et al, 2007) (Figure 10).

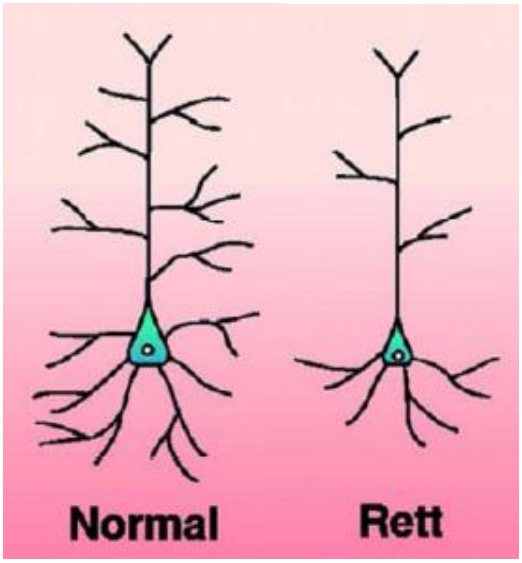


Figure 10: Schematic representation of pyramidal neurons from control and Rett brains.

Presynaptically, lack of MeCP2 is associated with an abnormal number of axons (Belichenko et al, 2009a) and a defect in axonal targeting (Belichenko et al, 2009b; Matarazzo et al, 2004; Palmer et al, 2008). Overall, the structural defects described at the synapse would suggest that loss of MeCP2 triggers alterations in the functioning of the synapses and, consequently, of the neuronal networks.

Analysis of neurotransmission associated with loss of MeCP2 provides further evidence for synapse dysfunction. Postmortem analysis in RTT brains showed altered levels of neurotransmitters such as glutamate and biogenic amines as well as changes in the abundance of some neurotransmitter receptors. In mice, reduced levels of serotonin (5- hydroxytryptamine), adrenaline, and dopamine have been found in the MeCP2-null brain (Ide et al, 2005; Isoda et al, 2010; Samaco et al, 2009; Santos et al, 2010). Analysis of spontaneous miniature excitatory and inhibitory postsynaptic currents indicated a shift in the excitatory/inhibitory (E/I) balance, with increased excitatory and decreased inhibitory neurotransmission in the hippocampus and cortex (Chao et al, 2007; Dani et al, 2005; Nelson et al, 2006; Wood & Shepherd, 2010; Zhang & Minassian, 2008). This is supported by data showing pre- and postsynaptic defects of GABA and therefore inhibitory, neurotransmission in the brainstem (Medrihan et al, 2008). Consistent with the idea of disturbance of the E/I balance, long-term potentiation (LTP) is also altered in the hippocampus of symptomatic MeCP2-deficient mice (Asaka et al, 2006; Guy et al, 2007; Weng et al, 2011). These data, added to the morphological studies, imply that loss of MeCP2 causes malfunction of numerous synapses throughout the brain, which creates less efficient neuronal networks and gives rise to RTT-like phenotypes.

1.3.2 MeCP2 genes target

The identification of MeCP2 genes target has always been one of the main objectives of many researchers. This will allow you to better understand the correlation between the presence of MeCP2 mutations and clinical manifestations of RTT and to think about how to make up for excess or deficiency of certain

proteins in these patients. Many studies with microarrays, with which it is possible to simultaneously evaluate the expression of a large number of genes, have contributed to uniquely identify a few target genes (Chahrour & Zoghbi, 2007) (Figure 11).

Gene	Function	References
Bdnf	neuronal development and survival	Chen et al., Martinowich et al.
xHairy2a	neuronal repressor	Stancheva et al.
DLX5/ Dlx5	neuronal transcription factor	Horike et al.
Sgk1	hormone signaling	Nuber et al.
Fkbp5	hormone signaling	Nuber et al.
Uqcrc1	mitochondrial respiratory chain	Kriaucionis et al.
ID1-3/ Id1-3	neuronal transcription factors	Peddada et al.
FXYD1/ Fxyd1	ion channel regulator	Deng et al.
IGFBP3/ Igfbp3	hormone signaling	Itoh et al.
Crh	neuropeptide	McGill et al.
UBE3A	ubiquitin ligase	Samaco et al.
GABRB3	GABA-A receptor	Samaco et al.

Figure 11: MeCP2 Target Genes.

In 2003, BDNF has been identified as the first MeCP2 target in mammals (Chen et al, 2003; Martinowich et al, 2003). BDNF is a neurotrophin essential for survival, growth, differentiation and maintenance of neurons during development (Ghosh et al, 1994). In the brain, it is active in the hippocampus, cortex, and basal forebrain-areas vital to learning, memory, and higher thinking (Yamada & Nabeshima, 2003). BDNF itself is important for long-term memory (Bekinschtein et al, 2008).

The role of BDNF in the pathogenesis of RTT is still unknown and it is unknown how the absence of MeCP2 results in a malfunction of BDNF and if this contributes to the neurological patients phenotype. In cultured neonatal cortical neurons, basal BDNF transcription is repressed by MeCP2 in the absence of neuronal activity, but activity-dependent upregulation of BDNF is unaffected by MeCP2 deletion (Chen et al, 2003). Chang and colleagues have shown that BDNF protein level in the whole-brain lysate in MeCP2 mutant mice was decreased to about 70% of the wild-type level (Chang et al, 2006). To further investigate the in vivo role of BDNF in RTT, BDNF expression has been manipulated in the postnatal brains of MeCP2-deficient mice and discovered that deleting BDNF from the MeCP2 mutant brain resulted in an earlier onset/accelerated disease progression, whereas overexpressing BDNF in the MeCP2 mutant brain led to later onset/slower disease progression. So they demonstrated in vivo a functional interaction between MeCP2 and BDNF and suggested that RTT may be a human disease that is partially mediated through BDNF.

Recently, Zhou and colleagues demonstrated that the phosphorylation of a specific amino acid residue S421 of MeCP2 controls the ability of the protein to regulate dendritic patterning, spine morphogenesis and the activity dependent induction of Bdnf transcription (Zhou et al, 2006). These findings suggest that, by triggering MeCP2 phosphorylation, neuronal activity regulates a program of gene expression that mediates neuronal connettivity in the nervous system.

1.4 Rett Mouse Models

With the discovery of the gene that causes the Rett Syndrome, several MeCP2 deficient mice models of RTT have been developed. Their creation has allowed examine the relationship between molecular and anatomical changes of the disease and associated behavioral abnormalities. These models also represent an extraordinary tool to test therapeutic interventions that may lead to improved an eventual cure for this devastating disease (Stearns et al, 2007).

Several mouse models currently exist (Figure 12).

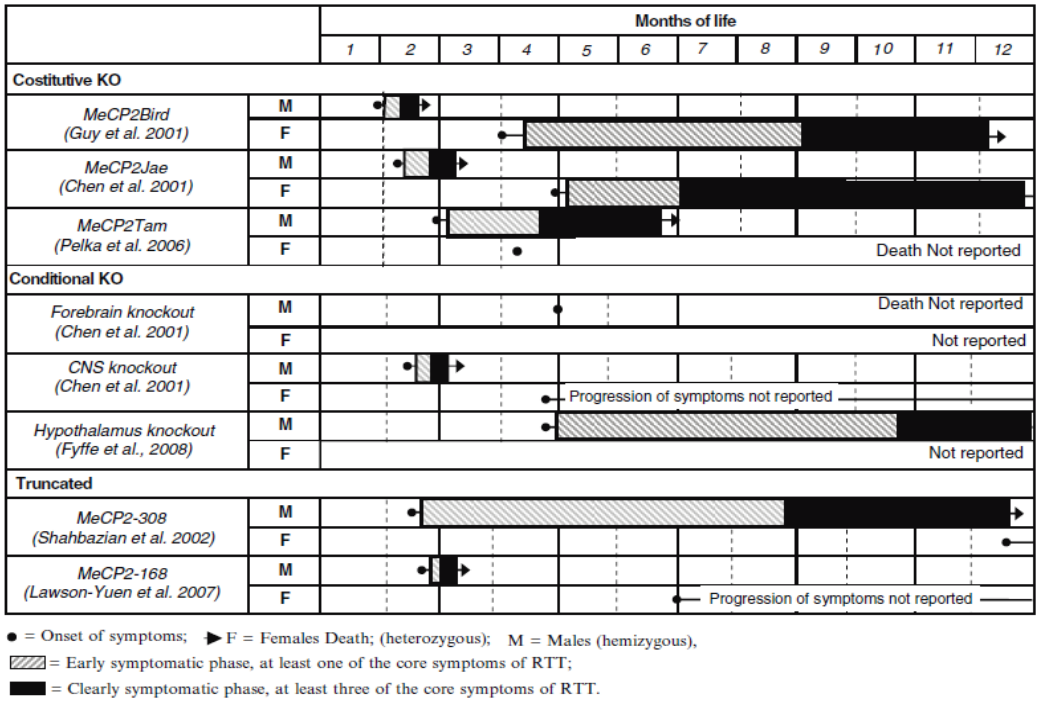


Figure 12: Lifespan and progression of symptoms of selected mouse models.

Three constitutive knockout models result in the loss of functional MeCP2 protein and were created using the technique of Cre-loxP recombination (Nagy, 2000).

In Dr Bird's laboratory, mice with deletions of exons 3 and 4 of MeCP2 (*Mecp2*$^{tm1\text{-}1Bird}$,which comprises nearly the entire protein) were created (Guy et al, 2001). Dr Jaenisch's lab produced a MeCP2-deficient mice model in which was

deleted only exon 3 *(Mecp2*^{1lox}), which contains a large part of the MBD domain (Chen et al, 2001). Both models produce MeCP2-null mice and MeCP2-heterozygotes, and result in similar phenotypes. MeCP2-null male mice are apparently healthy at birth until 3 weeks of age. After this period, mice begin to show neurological symptoms like those observed in RTT patients: stiff and uncoordinated gait, hind limb clasping, and irregular breathing. Uneven wearing of the teeth and misalignment of the jaws are also observed. Testes of MeCP2-null males were always internal. Symptoms progression leads to weight loss and early death around 54 days. Brain architecture in null mice is grossly normal, although a slight decrease in the size and weight can be noticed in comparison with wild-type littermates. This is due to neurons compaction in hippocampus, cerebral cortex and cerebellum. Total brain weight was reduced 9 to 13% compared to wild-type controls. Cortical brain volume was decreased between 7 to 11% and hippocampal volume was decreased 8% (Belichenko et al, 2008). By comparison, an autopsy study of Rett girls found brain weight to be reduced 12 to 34% relative to age-matched controls (Jellinger et al, 1988). MeCP2$^{+/-}$ females mice are viable, fertile and grow normally until adulthood. At 3 months, they start showing hind limb clasping and by 6 months they show RTT phenotypes such as breathing irregularity and inertia. These results show that MeCP2-null mice can be a good model to study RTT because of delayed onset of neurological symptoms affecting posture, gait, breathing and spontaneous movements. In 2006, Dr Patrick Tam generated mice with a targeted deletion of the methyl binding domain (MBD) resulting in complete loss of MeCP2 protein *(Mecp2*^{tm1Tam}, (Pelka et al, 2006). MeCP2^{tm1Tam} phenotype is comparable with that of the Jaenisch and

Bird's mice phenotype. In addition, at 8 to 10 weeks after birth, they display reduced level of anxiety, locomotors activity and cerebellar learning (Figure 13).

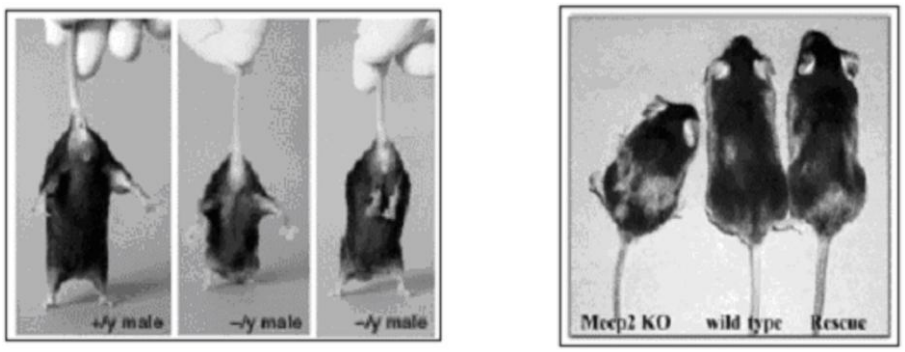


Figure 13: Rett mice phenotype.

In the fourth model, the MeCP2 protein is truncated after codon 308 (*Mecp2*^{308}), retaining several key functional domains (Shahbazian et al, 2002a). In these mice the phenotype is milder than those seen previously. MeCP2$^{308/y}$ are normal until 6 weeks and then they develop symptoms like tremors, kyphosis, learning and memory deficits, social behavior abnormalities, etc. Heterozygote females MeCP2$^{308/+}$ have impaired motor features starting at 35-39 weeks after birth. As in the case of human female patients, these mice show phenotypic variability due to the XCI. MeCP2-deficient models are reminiscent of RTT in that autonomic misregulation occurs (tremor, breathing irregularities, weight abnormalities), smaller brain and neuronal size is observed, posture and motor coordination are abnormal, and seizures may be present (Chen et al, 2001; Guy et al, 2001; Shahbazian et al, 2002a). Additionally, Rett mice show a similar developmental progression to Rett girls, appearing normal at birth, achieving motor and developmental milestones early on, and showing a delayed onset of symptoms. Mecp2-deficient mice show similar neurochemistry to Rett girls; cholinergic markers are low in the brains of Rett girls and Rett mice show phenotypic improvement in response to dietary cholinergic supplementation (Nag & Berger-

Sweeney, 2007; Wenk, 1997). Also, studies on Rett mice models are consistent with the view that RTT is mainly a synaptic disorder. Synaptic plasticity is impaired in Rett mice models, dendritic spines, the principal site of excitatory neurotransmission, are greatly reduced, and there appears to be an overall imbalance between excitation and inhibition (Armstrong, 2005; Dani et al, 2005). Recently neuron-specific or regional MeCP2 deletion studies reproduce some aspects of Rett syndrome. Has been demonstrated that loss of MeCP2 from dopaminergic neurons causes motor incoordination whereas loss from serotoninergic neurons leads to increased aggression (Samaco et al, 2009); furthermore the loss of MeCP2 from amygdale impairs amygdale-dependent learning and memory (Adachi et al, 2009) and MeCP2 deletion in hypothalamic Sim1-expressing neurons leads to alterations in feeding behavior, aggression and stress response (Fyffe et al, 2008). Postnatal loss of MeCP2 from forebrain excitatory neurons produces motor incoordination, increased anxiety-like behaviours and impaired fear conditioning and social behavior (Gemelli et al, 2006). In the last year Lioy and colleagues showed that in globally MeCP2-deficient mice, reexpression of MeCP2 preferentially in astrocytes significantly improved locomotion and anxiety levels, restored respiratory abnormalities to a normal pattern, and greatly prolonged life span compared to globally null mice. Furthermore, restoration of MeCP2 in the mutant astrocytes exerted a non-cell-autonomous positive effect on mutant neurons in vivo, restoring normal dendritic morphology and increasing levels of the excitatory glutamate transporter VGLUT1. Finally, they concluded their study showed that glia, like neurons, are integral components of the neuropathology of Rett syndrome, and supported the targeting of glia as a strategy for improving the associated symptoms.

1.5 Oxidative stress in Rett Syndrome

The antioxidant enzymatic system is one of the most important free radical detoxification mechanisms. The enzymes act in equilibrium, and any unbalance of this system may provoke oxygen-derived free radical generation. Free radicals are extremely reactive molecules that can disrupt lipid cell membranes, destroy cell enzyme functions, alter DNA, and lead to cell death (Shadid et al, 1998; Thomas & Aust, 1985). Free radicals appear to play a role in the pathogenesis of Rett syndrome (Sofic et al, 1987).

There is an increasing amount of experimental evidence that oxidative stress caused by oxygen free radicals is involved in the neuropathology of several neurodevelopmental and neurodegenerative disorders, as well as in stroke and seizures (Coyle & Puttfarcken, 1993).

A growing body of evidence supported the association between oxidative stress and stereotyped movements in different animal models and in patients affected by neurological disorders such as obsessive compulsive disorders or autism (Ghanizadeh, 2011; Ghanizadeh, 2012; Guldenpfennig et al, 2011).

Elevated cerebrospinal fluid glutamate has been found in Rett syndrome (Hamberger et al, 1992). The neurotransmitter glutamate can provoke oxidative stress in cells (Coyle & Puttfarcken, 1993). Moreover, a postmortem study of a case with RTT showed a severe reduction of ascorbic acid and reduced glutathione levels in most brain regions (Sofic et al, 1987). Ascorbic acid and glutathione play an important role in the antioxidant control system, preventing excessive accumulation of free radicals in living tissue. The authors suggested that the impairment of these defense mechanisms could indicate a process of progressive neurological illness with mental retardation and delayed motor

development. High levels of malondialdehyde reflect peroxidative damage of bio-membranes that might contribute to progressive dementia, impaired motor function, behavioral changes, and seizures (Buss & Winterbourn, 2002; Dalle-Donne et al, 2003). Any relationship was found between malondialdehyde levels and the clinical stages of pathology in a large number of patients. However, malondialdehyde levels were significantly lower in patients with moderate phenotype compared with patients with severe clinical phenotype. These results indicate that free radicals generated from oxidation reactions might contribute to the pathogenesis of Rett syndrome.

Formichi et al. (Formichi et al, 1998) found low serum vitamin E levels in nine of 28 patients with RTT. These results indicated that the oxidative free radical metabolism might be impaired in some patients with Rett syndrome.

Interestingly, Brain-derived neurotrophic factor (BDNF), a MeCP2 gene target, that is down-regulated both at the RNA and protein levels in MeCP2-null mice, can protect neurons against oxidative damage resulting from different neuropathologic insults(Mattson, 2002). Is reasonable to assume that oxidative stress detected in Rett syndrome is at least partly due to decreased levels of BDNF. Likewise, the cAMP-responsive-element binding protein (CREB) is recently reported as another molecular target of MeCP2 (Chahrour et al, 2008), can produce a lipid peroxidation-induced differential regulation thought to play a role in neurodegenerative disease such as Alzheimer disease (Pugazhenthi et al, 2006).

1.6 Vascular Function

While the neurological phenotype of the Rett syndrome has been well-characterized in animal models and in humans, few studies focused on alterations in the cardiovascular system of MeCP2 deficient mice (Bissonnette et al, 2007; McCauley et al, 2011), but nobody specifically investigated the vascular functions in this pathology.

An important role in the vascular functions is played by the endothelium: a thin layer of cells that serves not merely as a passive barrier between flowing blood and the vascular wall but uses this strategic location to maintain vascular homeostasis. The healthy endothelium is able to respond to physical and chemical signals by production of a wide range of factors that regulate vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. The importance of the endothelium was first recognized by its effect on vascular tone. Pioneering experiments by Furchgott and Zawadzki1 showed that the presence of intact endothelium was essential for acetylcholine (ACh) to induce dilation of isolated arteries. In contrast, if the endothelium was removed, the arteries constricted in response to ACh. Subsequent studies revealed that ACh stimulated the release of a potent vasodilating substance by the endothelium, identified as nitric oxide (NO) (Furchgott, 1996; Ignarro et al, 1987). NO is probably the most important and the best characterized mediator, and its intrinsic vasodilator function is commonly used as a surrogate index of endothelial function. It is generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin (Forstermann & Munzel, 2006). NO produces vasodilation primarily by activating guanylyl cyclase in vascular smooth muscle cells, which increases intracellular

concentrations of cyclic-3',5'-guanosine monophosphate (cGMP) (Arnold et al, 1977). cGMP in turn acts as a second messenger, activating cGMP-dependent protein kinase, which decreases cytosolic calcium concentration and modulates ion channel function leading to relaxation of vascular smooth muscle cells (Lincoln & Cornwell, 1993). NO inhibits platelet aggregation and adhesion by a guanylyl-cyclase mechanism (Radomski et al, 1990). Endothelium-devived NO is an inhibitor of leucocyte adhesion at the vessel wall (Kubes et al, 1991), and has anti-mitogenic effects on vascular smooth muscle cells (Garg & Hassid, 1989) (Figure 14). Thus, it is clear how in normal vascular physiology, NO plays a key role to maintain the vascular wall in a quiescent state by inhibition of inflammation, cellular proliferation, and thrombosis. Normal NO release opposes the vasoconstrictor responses to clinically relevant stimuli including catecholamines and serotonin (Golino et al, 1991; Vita et al, 1992). NOS inhibition is associated with increased systemic blood pressure, decreased blood flow responses to exercise and local ischemia (Gilligan et al, 1994; Meredith et al, 1996), and shortened bleeding time.

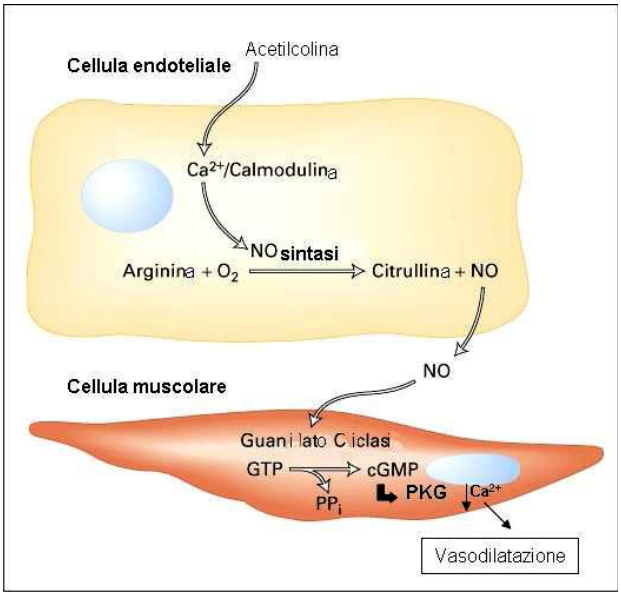


Figure 14: Nitric oxide in the regulation of vasodilation.

Failure of endothelium to elicit NO-mediated vasodilation is caused by reduced bioavailability of endothelium-derived NO due to either decreased formation or accelerated degradation. A large body of evidence shows that accelerated degradation of NO by reactive oxidant species (ROS) accounts for a large proportion of reduced NO bioavailability and endothelial dysfunction in vascular disease states. Both excess generation of ROS including superoxide anion and oxidized LDL cholesterol and decreased antioxidant defense mechanisms contribute to enhanced degradation of NO (Kojda & Harrison, 1999).

Superoxide anion can directly inactivate NO by reacting very rapidly to form peroxinitrite, which is vasoinactive and a powerful, damaging oxidant more stable than either superoxide or NO (Gryglewski et al, 1986; Rubanyi & Vanhoutte, 1986). In human blood vessels, increased superoxide production is also associated with impaired NO-mediated vasorelaxation (Landmesser et al, 2000).

Another consequence of increased production of ROS in the vasculature is lipid peroxidation. Oxidized LDL is cytotoxic to endothelial cells. It can react with NO directly and eliminate its biological activity or interfere with signal transduction and receptor-dependent stimulation of NOS activity and with activation of guanylyl cyclase.

Given the relationship between increased oxidative stress in the vasculature and impaired endothelial vasodilator function, researchers considered the possibility that augmenting antioxidant defenses would have a beneficial effect. For example, Vitamin E supplementation improves the bioactivity of endothelium-derived NO in hypercholesterolemia and in diabetes mellitus (Andersson et al, 1994; Keegan et al, 1995). Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension (Taddei et al, 1998).

Reduced NO formation may also contribute to endothelial dysfunction. Although the NO-producing enzyme eNOS is constitutively expressed by endothelial cells, its expression is subject to modulation by shear stress, atherogenic lipoproteins, and cytochines. Altered eNOS expression may affect NO synthesis. Several pathways can regulate eNOS expression and function.

In vitro data demonstrate a selective and differential presence of MeCP2 at promoter level of eNOS endothelial cells and vascular smooth muscle cells (Chan et al, 2004; Fish et al, 2005). These findings are in line with data in humans, reporting elevated plasma levels of oxidative stress in MeCP2-RTT patients, together with a decreased superoxide dismutase activity, and increased markers of lipid peroxidation (De Felice et al, 2009; Sierra et al, 2001).

1.7 Curcumin

Natural plant products have been used throughout human history for various purposes and its role in human healthcare cannot be underestimated. In developing countries, approximate 80% of individuals depend primarily on natural products to meet their healthcare needs.

Curcumin ($C_{21}H_{20}O_6$) is a natural product in use for thousands of years. It is an active constituent of *Curcuma longa Linn* (Zingiberacee) a perennial herb that grows in South and Southeast Asia. The main component turmeric is from the rhizome, or root of the plant. The 2% of curcumin is extracted in 95% ethanol for 24 hours, then filter and dry. Curcumin is classified as a polyphenol compound that gives turmeric its bright yellow color. Figure 15 shows the physical look of Curcuma longa and curcumin extract.

Figure 15: The physical look of Curcuma longa: plant and powder.

In 1910 Milobedzka and colleagues described its chemical structure and shown to be diferuloylmethane. The major curcuminoids found in turmeric (about 3-5%) include demethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III) and recently was identified cyclocurcumin (Figure 16).

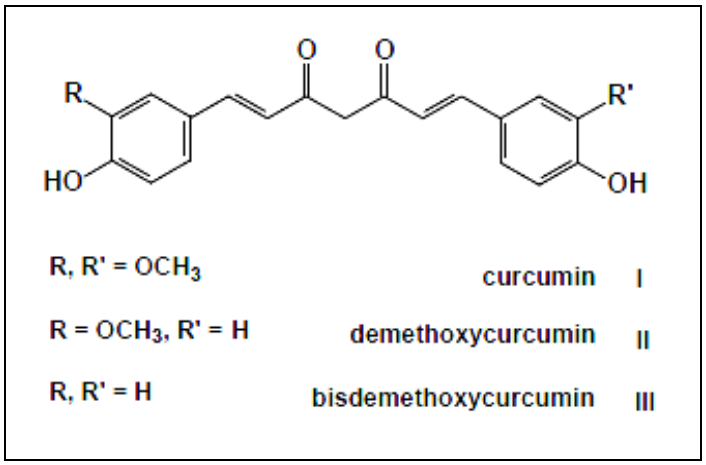


Figure 16: Chemical structure of the major curcuminoids.

Turmeric is used as a dietary spice, coloring agent in foods and textiles, and as treatment for a wide variety of aliments. Curcumin holds a high place in ayurvedic medicine as a "cleanser of the body," (Ammon & Wahl, 1991) and today, science has documented several diseased conditions that can be healed by the active ingredients of turmeric (Mishra & Palanivelu, 2008; Nair et al, 2010) . Several studies evidenced that curcumin possesses anti-oxidant, anti-inflammatory, neuroprotective, anti-cancer, antiviral, antibacterial and antifungal properties and has been found that this phytochemical potentially prevents and treats cardiovascular, pulmonary, metabolic, neurodegenerative, autoimmune and other chronic illness like neoplastic diseases (Aggarwal & Harikumar, 2009; Sandur et al, 2007).

1.7.1 Metabolism and Bioavailability

Clinical trials have shown that curcumin amount required for disease management i.e. minimum of 8 g/day and it is safe even at high doses (12 g/day) in humans but exhibit poor bioavailability (Lao et al, 2006) due to insolubility in water, poor absorption and rapid metabolism.

It was shown that oral consumption of 1 g/kg curcumin in rats resulted in approximately 75% being excreted in the feces and only traces appeared in the urine (Wahlstrom & Blennow, 1978), whereas intra-peritoneal administration accounted for similar levels of fecal excretion of curcumin, with only 11% found in bile (Holder et al, 1978) suggesting poor absorption of curcumin from the intestine. Numerous studies revealed that curcumin is bio-transformed to dihydrocurcumin and tetrahydrocurcumin and subsequently, these products are converted to monoglucuronide conjugates (Pan et al, 1999). These metabolites are detected in plasma or serum following oral consumption. It was reported that the main biliary metabolites of curcumin are glucuronide conjugates of tetrahydrocurcumin (THC) and hexahydrocurcumin (Holder et al, 1978). Curcumin metabolites may not have the same biological activity as the parent compound. Recently, a clinical trial conducted in the UK found that plasma concentrations of curcumin, curcumin sulfate, and curcumin glucuronide were in the range of 10 nanomoles/liter (0.01 micromole/liter) one hour after a 3.6 g oral dose of curcumin (Sharma et al, 2004). Curcumin and its metabolites could not be detected in plasma at doses lower than 3.6 g/day. Surprisingly, analysis of urine suggested the presence of curcumin and its conjugates in all samples from patients consuming this dose.

There are evidences that orally administered curcumin accumulates in gastrointestinal tissues. For instance, when colorectal cancer patients took 3.6 g/day of curcumin orally for seven days prior to surgery, curcumin was detected in malignant and normal colorectal tissue and only trace were found in the peripheral circulation (Garcea et al, 2005). In contrast, curcumin was not detected in the liver tissue of patients with liver metastases of colorectal cancer after the same oral dose of curcumin and also in this case only trace were found in peripheral circulation (Garcea et al, 2004), suggesting that oral curcumin administration may not effectively deliver curcumin to tissues outside the gastrointestinal tract. To improve the bioavailability of curcumin, numerous approaches have been undertaken. These approaches involve the use of adjuvant like piperine that interferes with glucuronidation, the use of liposomal, nanoparticles, phospholipid complexes and structural analogues of curcumin.

1.7.2 Biological Activity

Curcumin exhibits strong antioxidant activity, comparable to vitamins C and E and appears to have a significant potential in the treatment of multiple diseases that are the result of oxidative stress. In fact, this yellow pigment effectively scavenges reactive oxygen species including superoxide anion radicals, hydroxyl radicals (Reddy & Lokesh, 1994) and reactive nitrogen species (Sreejayan & Rao, 1997; Unnikrishnan & Rao, 1995). It also inhibits lipid peroxidation in different animal models (Reddy & Lokesh, 1992; Sreejayan & Rao, 1994) and induces antioxidant and cytoprotective enzymes (Dinkova-Kostova & Talalay, 2008; Motterlini et al, 2000). For example, curcumin protectes against homocysteine-induced oxidative stress, decreasing lipid peroxidation and improves learning and

memory process that is impaired by homocysteine (Ataie et al, 2010). Vascular endothelial cells treated with curcumin prevent oxidant mediated injury by increase heme oxygenase production (Motterlini et al, 2000). In addition to its direct ability to attenuate the reactivity of oxygen free radical species, curcumin may function indirectly as antioxidant by inhibiting the activity of inflammatory enzymes or by enhancing the synthesis of glutathione, the most important endogenous antioxidant (Piper et al, 1998).

Several *in vivo* and *in vitro* studies reported the benefical effects of curcumin on the brain. For example, (Rajakumar & Rao, 1994) showed the curcumin inhibition of lipid peroxidation induced by ferrous ions, ferric ascorbate and ferric-ADP-ascorbate in brain homogenates. Has been showed in the brain of ethanol intoxicated rats, a significant curcumin-induced reversal of lipid peroxidation, and an enhancement of glutathione. Moreover, curcumin reduces oxidative damage on Alzheimer pathology. In fact curcumin inhibits amyloid beta oligomer formation (Yang et al, 2005). In animal models of Alzheimer's disease, dietary curcumin is effective in significantly lowering principal index of inflammation such as the proinflammatory cytokine IL-1β, GFAP, an astrocytic marker associated with injury and inflammatory processes and PT immunolabeling of microglia in cortical and hippocampal neuronal layers; reduces oxidative damage and decreases overall insoluble amyloid, soluble amyloid, and plaque burden (Frautschy et al, 2001; Lim et al, 2001; Pan et al, 2008). It is not known whether curcumin taken orally can cross the blood brain barrier or inhibit the progression of Alzheimer’s disease in humans. As a result of the promising findings in animal models, clinical trials of oral curcumin supplementation in patients with early Alzheimer’s disease are under way. The results of a 6-month trial in 27 patients

with Alzheimer's disease found that oral supplementation with up to 4 g/day of curcumin is safe (Baum et al, 2008). Recent studies have also shown that a diet with curcumin improves the inflammation associated with obesity and diabetes in a mouse model that shows obesity to insulin resistance as well as significantly reduced macrophage infiltration of adipose tissue, inflammatory and metabolic shifts prevent associated with obesity and improve glycemic control mouse models of diabetes type 2 (Weisberg et al, 2008). Is known that oxidative stress alters the neuronal function and plasticity after traumatic brain damage. Curcumin with its antioxidant activity reduces oxidative damage and normalizes the levels of BDNF and synapsin I, which is a phosphoprotein involved in the release of neurotransmitter, in stretching and maintenance of axonal synaptic contacts. Also normalizes the levels of CRE, a transcription factor involved in learning and memory and important modulator of gene expression induced by BDNF (Wu et al, 2006). Pretreatment of rat cortical neurons exposed to 10 μM glutamate for 24 hours, with curcumin reversed the BDNF expression and cell viability in a dose- and time-dependent manner (Wang et al, 2008). Moreover Xu and colleagues investigated the effect of orally administered curcumin on behavior in a chronic stress model of depression in rats. This study suggests that the effects of chronic administration of curcumin on the behavior of chronic stressed rats may be related to the modulating effects of the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, through selective increase in BDNF in the frontal cortex and the hippocampus of the rats (Xu et al, 2006). These results underscore the antidepressant effect of curcumin and its role in stress-related disorders.

Interesting was the finding of Bishnoi that indicates the curcumin as a therapeutic drug for haloperidol-induced tardive dyskinesia. In particular in this hyperkinetic

movement disorder curcumin is able to decrease oxidative damage and improves stereotypic movements caused by alteration in catecolamine metabolism (Bishnoi et al, 2008).

Has been demonstrate that curcumin is involved in chromatin remodeling, in particular in the suppression of histone acetylation. Li and colleagues hypothesized that this compound suppresses cardiac hypertrophy through the disruption of p300 histone acetyltransferase-dependent (p300-HAT dependent) transcriptional activation and downstream GATA4, NF-kappaB, and TGF-beta-Smad signaling pathways (Li et al, 2008).

It is also worth mentioning that given its activity on histone deacetylases (HDAC) (Chen et al, 2007), curcumin may be considered an epigenetic drug, this being of potential relevance in the absence of a correct MeCP2 based chromatin remodelling, like that found in the RTT patients.

AIM OF THE STUDY

In the classic form of Rett Syndrome, after a period of 6-8 months of apparently normal development, girls present a developmental arrest followed by a regression, with loss of speech and purposeful hand use accompanied by a reduction in interpersonal contact, stereotypic hand activities, EEG abnormalities and microcephaly. Other somatic and neurological symptoms include sleep disorder, muscular rigidity and cold and blue extremities to indicate peripheral circulation problems. It is not known whether RTT is associated to vascular abnormalities in peripheral microcirculation and dissecting the vascular function is feasible in human RTT patients. We have focused our studies on the vascular system functionality of MeCP2 null male mice at 50 days post birth, symptomatic MeCP2 heterozygous females and wild type littermates, using the pressured myograph system. Furthermore, since no effective pharmacological treatment is as yet available for this disorder, we investigated the effects of curcumin, a potent anti-oxidant and anti-inflammatory polyphenol with cardiovascular protective effects. We studied the effect of curcumin treatment on vascular functionality, molecular expression, behavior and motor activity of MeCP2 heterozygous adult females. They were included in this work because they have residual levels of MeCP2 with a mosaic expression due to the inactivation of the X chromosome, live longer than males, gradually over the time they develop motor deficits and stereotyped behaviors and for these phenotypic characteristics may be subject to complex behavioral tests. In Figure 17 is displayed this animals phenotype that is characterized by stereotypic behavior (crossing of the hind legs), abnormal breathing, tremors, changes in hair, hypoactivity, in contrast to wild-type animals, MeCP2 knockout if held by the tail show the reflection of placement of the legs.

Figure 17: Characteristic phenotype of Rett female mouse.

MATERIALS AND METHODS

3.1 Animals and genotyping

All experiments were carried out in accordance with the directives of Council of European Communities (86/609/EEC) and approved by Italian Ministry of Health for the care and use of laboratories. The animals used for experiments were derived from heterozygous B6.129SF1-*MeCP2*$^{tm1/Jae}$ B6 knock-out females (MeCP2$^{+/-}$) (Chen et al, 2001). The MeCP2$^{tm1/Jae}$ male mice exhibit a significant loss of body weight, body tremor and shaking paw by the fifth week, while heterozygous mutant females seem normal for the first four months when begin to show pathological symptoms such as reduced activity, ataxic gait, piloerection, stereotyped movements and heavy breathing.

Heterozygous females were originally crossed to C57BL6 for two generation, followed by breeding among offspring of the same generation with breeder changes and were maintained on a mixed background. Mixed background reduced mortality and was necessary to obtain the high numbers of mice required by extensive analysis. Age-matched littermates were used in all experiments to control for possible effects of genetic background unrelated to the *MeCP2* mutation (Boggio et al, 2010; Lonetti et al, 2010; Ricciardi et al, 2011). Mutant and wild-type mice were reared in standard condition: housed in polycarbonate cages with water and food available *ad libitum*. The experimental rooms were kept on a 12:12 light:dark cycle with lights on from 6:00 AM to 6:00 PM. Ambient temperature was maintained at 21°C. Mice were genotyped by PCR. DNA extracted from the biopsy of a small piece of tail taken to P10. MeCP2 alleles identified by polymerase Chain Reaction (PCR) using two sets of primers. The first primer (5' primer: 5'-CAC CAC AGA AGT ACT ATG ATC) and the

second primer (3' primer: 3'-CTG ACA ATG AGC TTT CTT CTA) used to produce 250 bp to identify MeCP2 null allele. After amplification, the DNA left to run on 1,5% agarose gel electrophoresis containing ethidium bromide and examined under UV light to identify genotype.

3.2 Curcumin treatment

Six months old symptomatic MeCP2$^{+/-}$ females as well as the age-matched wild-type littermates were split into two equal and homogeneous experimental groups (five animals for each group) on the bases of age, weight, and coordination capability (Rotarod test). The curcumin treated animals were feed, for 21 days with 5% curcumin (5 g curcumin in 100 g pellets for rodents) and they weighed a time at week from the first day of treatment. The pellets were softened with a small amount of water so they can be unmade and added the appropriate percentage of turmeric powder. After that, with the help of a syringe, were reconstituted in their original form. The same procedure was also performed for the food of controls group. Water was provided *ad libitum* instead. The administration was performed every two days and each time has been marked the amount of food consumed and supplied to the two groups. All behavioural experiments were conducted during the treatments period and three weeks after animals were sacrificed and mesenteric artery, aorta, cerebral cortex and blood were extracted for molecular and functional experiments.

3.3 Pressure Myograph System

The Pressure myograph system is used to measure the physiological function and properties of small arteries, veins and other vessels. In this system, vessels retain many of their in vivo characteristics. In pressure myography, an intact small segment of an artery or vein is mounted onto two small glass cannulae and pressurized to a suitable transmural pressure. This near physiological condition permits the investigation of intrinsic (myogenic) responses which can be extrapolated to the in vivo behaviour of the entire vascular bed (autoregulation). Both constriction and dilation can be readily measured as changes in diameter of the preparation via digital video edge-detection. Since intrinsic myogenic constriction is present, the role and function of the endothelium for this phenomenon can be studied.

For reactivity experiments a third-order branch of the mesenteric arterial tree (≈2 mm in length) was dissected and isolated using a stereo microscope. The vessel was mounted on two glass microcannule in a microvessel chamber and secured with a ligature (Figure 18).

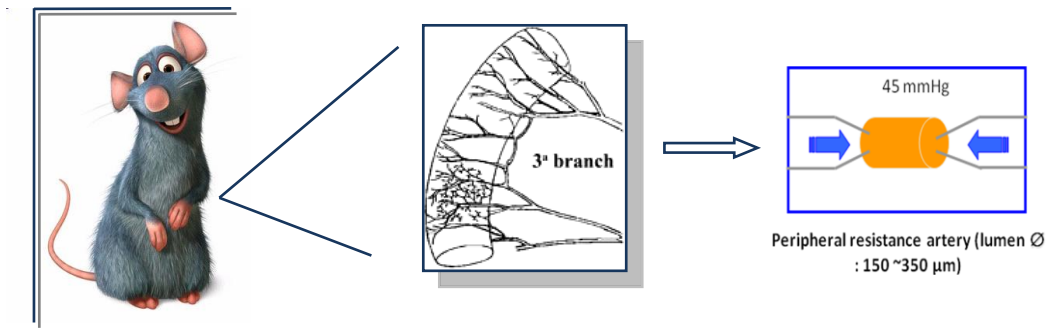


Figure 18: Representation of mouse third-order branch of mesenteric arterioles isolated and mounted on two glass microcannule.

The proximal cannula was connected to a pressure servo controller and the distal cannula was closed with a stopcock. The chamber was mounted on a stage of a microscope connected to a video camera and then to a personal computer equipped with a video dimensioning software. The vessel was visualized on the computer monitor and the internal diameter was measured by adjusting the guides superimposed by the software (Figure 19).

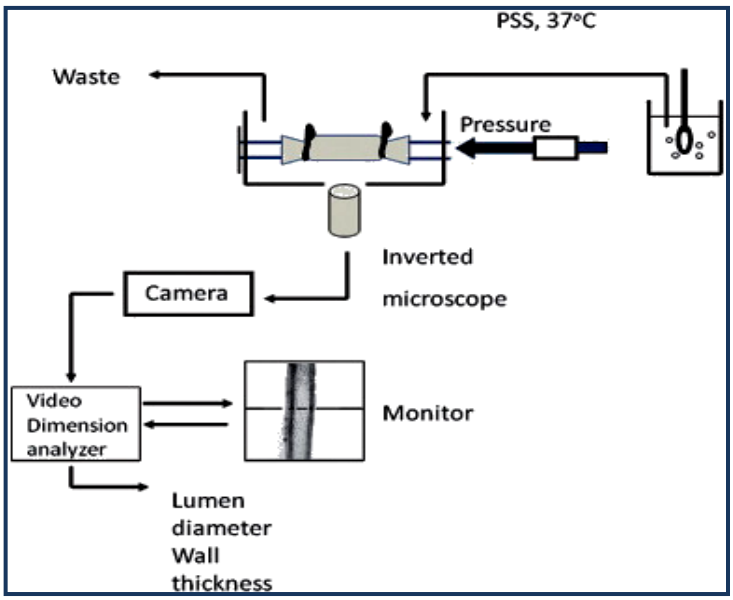


Figure 19: Schematic representation of pressure myograph system.

In our experiments, in particular, the vessels were equilibrated for 60 minutes under constant intraluminal pressure (45 mmHg) in warmed (37°C) and bubbled (95% air and 5% CO_2) physiological salt solution, which contained NaCl 120 mM, $NaHCO_3$ 25 mM, KCl 4.7 mM, KH_2PO_4 1.18 mM, $MgSO_4$ 1.18 mM, $CaCl_2$ 2.5 mM, EDTA 0.026 mM, and glucose 5.5 mM (pH 7.4). Vessels were considered viable and used if they constricted ≈70% of their resting lumen

diameter in response to an extraluminal application of high-potassium (125 KCl mM) physiological salt solution containing 100 μM of noradrenaline (NA).

In small arteries from all groups of animals, endothelium-dependent and -independent relaxations were assessed by measuring the dilatory responses of mesenteric arteries to cumulative concentrations of acetylcholine (0.001-100 μM, Sigma Chemicals) and sodium nitroprusside (0.01-100 μM, Sigma), respectively, in vessels precontracted with norepinephrine (10 μM). To evaluate NO availability, a curve to acetylcholine was constructed after 30-min preincubation with the NOS inhibitor L-NAME (100 μM, Sigma). Finally, to evaluate the influence of ROS, acetylcholine was repeated after 30-min preincubation with the antioxidant ascorbic acid (100 μM, Sigma).

3.4 Determination of Superoxide Anion Production in the Mesenteric Vessels

Dihydroethidium (DHE, Sigma) was used to detect superoxide anion production. This compound is a lipophilic cell-permeable dye that can be rapidly oxidized to a fluorescent marker product by O_2^- produced within the cell. The fluorescent product intercalates with DNA and stains the cell nucleus red.

Frozen mesenteric vessels were cut into 30-μm thick sections and placed on a glass slide. Three slides per segment were analyzed simultaneously after incubation with Krebs solution at 37°C for 30 minutes. Krebs-HEPES buffer containing 2 μM DHE was then applied to each section and incubated in a dark incubator at 37°C for 30 minutes. Ethidium fluorescence was detected with a 563-nm long-pass filter after excitation at 543 nm, and images were obtained with a Zeiss LSM 510 confocal microscope equipped with a krypton/argon laser. The percentage of arterial wall area stained with the red signal was estimated using an imaging software (McBiophotonics Image J; National Institutes of Health, Bethesda, MD). All experiments were performed on duplicate.

3.5 Measurements of Plasma Malonyldialdehyde Levels

Malondialdehyde is one of the several low molecular weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products, it can be measured by reaction with thiobarbituric acid (Ohkawa et al, 1979). The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening of monitoring lipid peroxidation in several types of samples including human and animal tissues and fluids. The blood was obtained by cardiac puncture in presence of 0,5 M EDTA and then isolated by centrifugation at 3000 rpm for 5minutes, pipetted off the top yellow plasma layer without disturb the white buffy layer and freezed at -80°C until experiment time. The plasma samples will be stable for one month while stored al -80°C. Systemic lipid peroxidation was quantified by measuring plasma malonyldialdehyde (MDA) levels by commercial kit (Cayman Chemical). The MDA-TBA adduct performed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions is measured colorimetrically. In particular, each sample (100 µl) was added with the same volume of SDS Solution and 4 ml Colour Reagent. The vials were boiled in water for one hour and then immediately place in ice bath to stop the reaction. After 10 minutes, vials were centrifuged for 10 minutes at 1600 g at 4°C. 150µl of each sample was loaded in the plate and read the absorbance at 530-540 nm. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted.

3.6 RNA extraction and Real Time PCR

Expression of mRNA for eNOS and iNOS was assessed by Real Time PCR. Total RNA was isolated from mesenteric vessels, aorta and cerebral cortex with Tripure Isolation Reagent (Roche Molecular Biochemicals). Briefly, 1 ml Tripure Isolation Reagent was added to tissue and then homogenized. Each homogenized sample was added with 0.2 ml of chloroform, shaked vigorously and incubated for 15 minutes at room temperature. To separate the solution in three phases (colorless aqueous phase containing RNA, white interphase and red organic phase containing protein and DNA; Figure 20) samples were centrifuged at 12000g for 15 minutes at 4°C. To precipitate the RNA the colorless aqueous phase was transferred in a new centrifuge tube and added with 0,5 ml isopropanol, mixed and centrifugated at 12000 g for 10 minutes at 4°C. The supernatant was discarded and pellet washed with 75% ethanol. Finally the RNA pellet was resuspended in diethylpyrocarbonate (DEPC)-trated RNase free water and incubated for 10 minutes at 55°C.

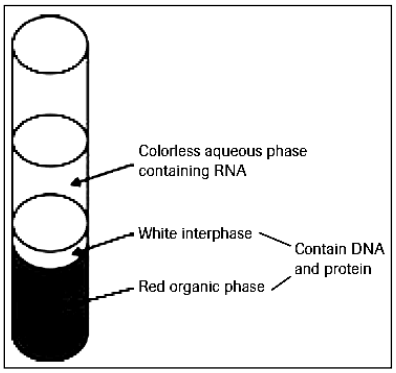


Figure 20: Representative diagram of the three phases of the solution: upper colorless aqueous phase contains RNA; white interphase and lower red organic phase contain DNA and protein.

The RNA was quantified by spectrophotometry and 1 µg used for the synthesis of first-strand cDNA trough High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as shown below.

Component	**Volume/Reaction (µl)**	**Thermal cycling condictions**
10X RT Buffer	2.0	**Step 1** : 25°C 10 min **Step 2**: 37°C 120 min **Step 3**: 85°C 5 sec **Step 4**: 4°C ∞
25X dNTP Mix (100mM)	0.8	
10X RT Random Primers	2.0	
MultiScribe Reverse Transcriptase	1.0	
Nuclease-free H2O	4.2	
Total per Reaction	10.0	

The reaction of quantitative real-time PCR was performed, on a rate equal to 1/10 of the RT reaction product, using Taqman Gene Expression Assays (Applied Biosystems) and the "delta-delta Ct" method. Sequences targeted by fluorescent probes were endothelial nitric oxide synthase (eNOS; Mm00435204_m1; Applied Biosystems) and inducible nitric oxide synthase (iNOS; Mm00440485_m1; Applied Biosystems) and the probe against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm 99999915_g1; Applied Biosystems) as housekeeping gene. The PCR conditions included 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15s 60°C for 1 min.

3.7 Immunostaining of eNOS

Small mesenteric arteries were immediately fixed in cold 4% paraformaldehyde after collection from small bowel loops and paraffin embedded at 56°C. Eight-micron-thick sections were serially mounted on glasses and sequentially treated as follows: 1% hydrogen peroxide in methanol for 30 minutes; microwave antigen retrieval (600 W in 10 mmol/L of sodium citrate; pH 6.0); normal goat serum (1:20; Dakopatts Glostrup, Denmark), rabbit anti-eNOS polyclonal antibody (sc-654, 1:100 overnight at 4°C, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), biotinylated anti-rabbit immunoglobulins, peroxidase-labeled streptavidin complex (Vector Laboratories, Burlingame California), 3,3-diaminobenzidine tetra-hydrochloride (DAB; Amresco, Solon, OH, USA), hematoxylin counterstaining. Negative controls were obtained by substituting the primary antibody with preimmune rabbit serum. Endogenous peroxidases and avidin-binding activity were assayed by incubating slides with DAB alone or with peroxidase-labeled streptavidin complex/DAB, respectively as previously reported (Virdis et al, 2007).

3.8 Rotating rod test (Rotarod)

The rotarod has been used to assess the ability of animals to balance on a rotating rod. As the speed of rotation was increased, it became more difficult for the animal to keep its balance. We tested the time it took the animal to fall under continuous acceleration from 4 to 40 rpm. The latency to fall off the rotating rod was used as an indication of motor coordination and balance. The experimental apparatus consisted of a rotating drum whose surface was manufactured to provide optimal grip for the animal. Separating disk divided the drum into five separate exercise lanes each suited for an individual animal (Figure 21). The mouse dropped onto the individual sensing platform below and the instrument automatically recorded the amount of time spent on the drum.Treated and untreated animals (Rett and wild-type) were tested before and after curcumin administration on rotarod apparatus. Mice performed five trials per day allowing a 5 minutes inter-trial interval. To habituate, mice have been transferred, in their home cages, from the holding room to the experimental room 30 minutes before the experimental section start.

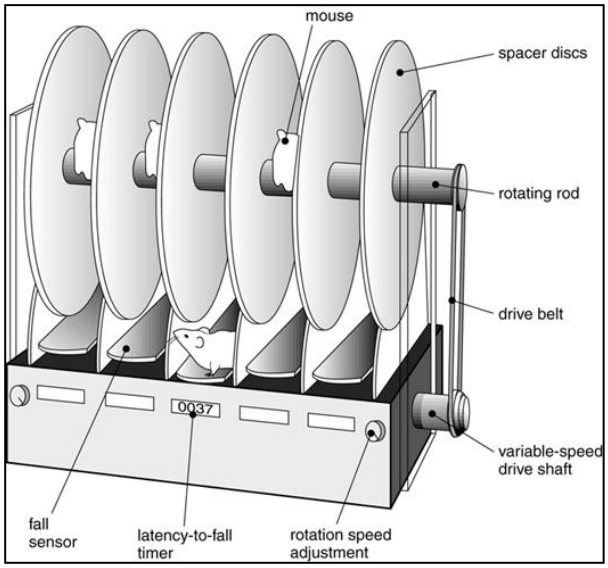


Figure 21: Schematic Rotarod experimental apparatus.

3.9 Behavioural observation

To test whether curcumin treatment caused behavioral changes in wild-type and RTT mice, they were divided into two groups and one was administered with the drug. Before and after treatment, at three different times of the day (morning 9 am, afternoon 1 pm and evening 6 pm) the animals were recorded with a camera in their cages. Each test session lasted two hours and the duration time of stereotyped movements (repetitive and involuntary movements that animals make, especially with the front legs, scratching the back up to injuring) and resting time (the time that the animals are sleeping or not moving) were measured. Cages were placed in the test room at least half an hour before the experiment so that the animals were not stressed in the new environment.

3.10 Open Field test

The open field test is a simple assessment used to determine general activity levels, gross locomotor activity and exploration habits in mice. It is based on the natural tendency of an animal to explore and avoidance reaction to protect itself, which make normal animals to spend more time in the corners and the periphery than in the centre (the most anxiogenic area).

The apparatus consisted of an arena square (60 x 60 x 30 cm) with transparent vinyl flooring material (lexan) and black walls. Under the floor there was a white card which was drawn a square (30 x 30 cm) at the center of the arena (Figure 22). The arena was connected to a camera that reproduced on a computer monitor the image of the apparatus.

Figure 22: Open Field arena square used for testing anxiety and exploratory drive.

The acquisition of the tracks was through a video tracking system Noldus (Ethovision XT, Wageningen, The Netherlands) that allowed the inclusion of

variables experimental (eg. name, treatment, sex, age, genotype). The system was able virtually to divide the arena into a "center" (center of the arena), a "periphery" (portion of the space between the edge of the arena and the central square), the "corners" (corners of the arena) and the "central point" (point located at the exact center arena) and to identify some parameters that allowed the program to consider the animal as the object of interest. Parameters recorded were: distance travelled in the whole arena and in the central and peripheral zones; percentage of time spent in the central zone and in peripheral zones; duration of the immobility (resting time in seconds) in the whole arena, in the periphery and in the center; number of entries in the central zone; latency to entry into the central zone.

Testing was conducted during the light phase of the cycle and the animals were transported in their cages and left undisturbed for 30 minutes before the test.

The apparatus cleaned with 10% alcohol and allowed time for it to dry. Each mouse was placed in the middle of a peripheral zone of the arena facing the wall and allowed to explore freely the apparatus for 5 minutes, with the experimenter out of the animal's sight. At the end of the run, animals were put back into their home cage. After each run, any faeces were removed and the arena was thoroughly wiped (water then alcohol 10 %). Indices of anxiety in animals were: decreased locomotor activity (basal locomotion), decreased stay at the center and increased anxiety behaviours such as defecation. The only time spent in the center could be used as a preliminary indication of anxiety: very anxious mice tend to avoid the center of the arena. The total activity tends to decrease over time and this is considered a measure habit of novelty apparatus.

3.11 Data Analysis

Results are presented as mean±SEM and analyzed by Student's *t* test or two-way repeated measures ANOVA where appropriate.

Data were analyzed using GraphPad 5 analysis software. The unpaired Student's *t* test used to make comparisons between groups used. The significance of the effects of factors and the differences between samples were estimated by analysis of variance (two-way repeated measures ANOVA). Data are presented as mean ± SEM and a value of $P<0.05$ was considered statistically significant.

RESULTS

4.1 Vascular reactivity on WT and Rett mice

A third-order branch of the mesenteric arterial tree was dissected and isolated using a stereo microscope. The vessel was mounted on two glass microcannule in a microvessel chamber and the mesenteric vessel resistance was investigated with pressurized myograph.

In mesenteric vessels from the WT group, the relaxation to increasing concentration of acetylcholine was preserved. This was significantly attenuated by L-NAME (a NOS inhibitor) and not affected by the antioxidant ascorbic acid (Figures 23A and 24).

On the contrary, vessels from MeCP2$^{+/-}$ female mice showed a significant ($P<0.001$) attenuated relaxation to acetylcholine. In this group, the inhibitory effect of L-NAME on relaxation to acetylcholine, although still evident at the maximal dose, was significantly blunted (Figures 23B and 24). In addition, ascorbic acid potentiated the relaxation to acetylcholine and restored the inhibitory effect of L-NAME (Figure 23B).

Investigating the mesenteric vessel resistance from MeCP2$^{y/-}$ male mice we evidenced as they were characterized by a further reduced relaxation to acetylcholine as compared to MeCP2$^{+/-}$ female mice ($P<0.05$), which was totally resistant to L-NAME. Ascorbic acid only in part enhanced the relaxation to acetylcholine and restored the inhibition by L-NAME (Figure 23C).

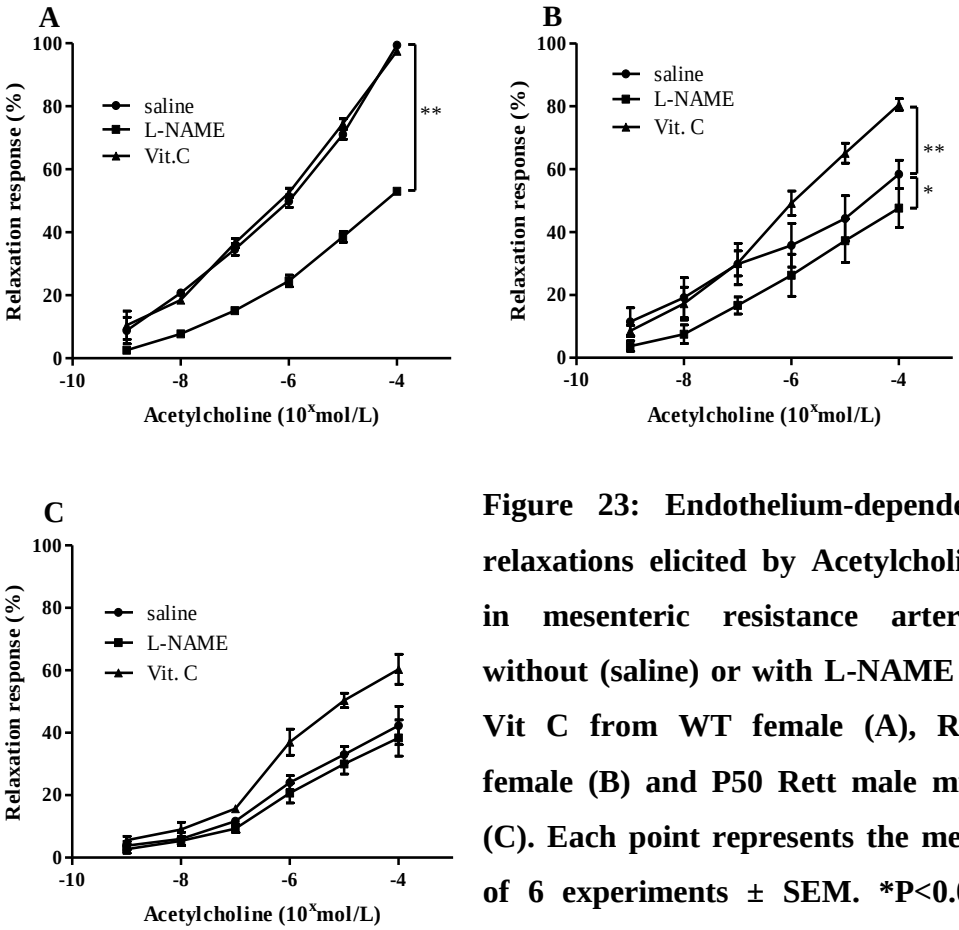


Figure 23: Endothelium-dependent relaxations elicited by Acetylcholine in mesenteric resistance arteries without (saline) or with L-NAME or Vit C from WT female (A), Rett female (B) and P50 Rett male mice (C). Each point represents the mean of 6 experiments ± SEM. *P<0.05, **P<0.01.

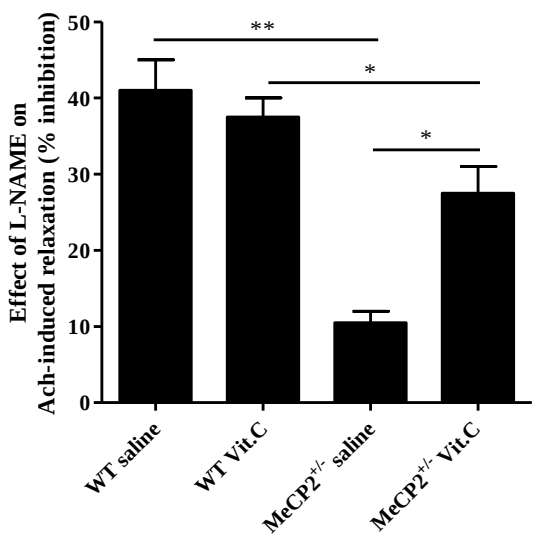


Figure 24: Inhibition by L-NAME on maximal response to acetylcholine in vessels from WT, MeCP2$^{+/-}$, and curcumin-treated MeCP2$^{+/-}$ mice, without (saline) or with ascorbic (asc) acid. Results are given as the mean of 6 experiments ± SEM. *P<0.05, **P<0.01.

At the same time, to test the endothelium-independent relaxation we incubated the mesenteric vessels of each group with sodium nitroprusside, and we found that the relaxation to this compound was similar in WT and MeCP2$^{+/-}$ animals (Emax: 97.2±0.8% and 97.8±0.9%, respectively), but it was significantly attenuated in MeCP2$^{y/-}$ mice (Emax: 80.4±1.1%; P<0.01 vs other groups) (Figure 25).

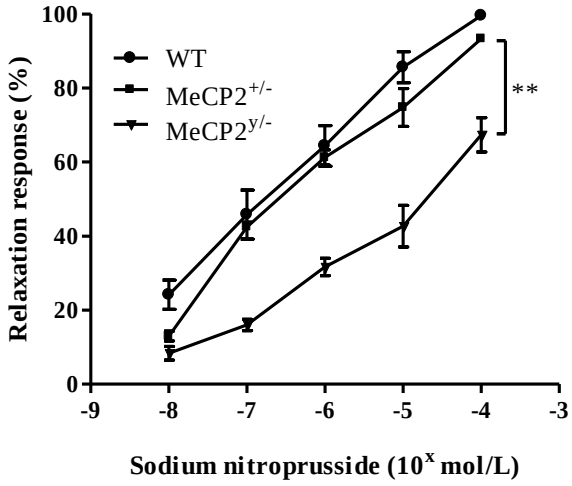


Figure 25: Vasorelaxation endothelium independent. Vascular response to increasing concentrations of sodium nitroprusside in WT and Rett female and male mice. **P<0.01.

4.2 Endothelial function: effect of curcumin

Rett female mice and wild type littermates were treated 21 days with curcumin 5% (w/w) in the diet.

After this period vascular functionality study highlighted that curcumin treatment dramatically potentiated, but nor normalized, the relaxation to acetylcholine in mesenteric vessels from MeCP2$^{+/-}$ mice, and restored the inhibitory effect of L-NAME on acetylcholine-induced relaxation (Figure 25). In such arteries, the vascular response to acetylcholine was no longer affected by ascorbic acid (Figure 26).

On the contrary, in vessels from WT mice, curcumin administration did not modify the relaxation to acetylcholine (E_{max}: 97.2±0.6%), the effect of ascorbic acid (E_{max}: 96.4±0.6%), or the inhibitory effect of L-NAME on acetylcholine (E_{max}: 58.3±0.9%; inhibition: -38.9±0.7%) were null.

Relaxations to sodium nitroprusside were not modified by curcumin administration in MeCP2$^{+/-}$ and WT (E_{max}: 97.2±0.8% and 97.8±0.9%, respectively).

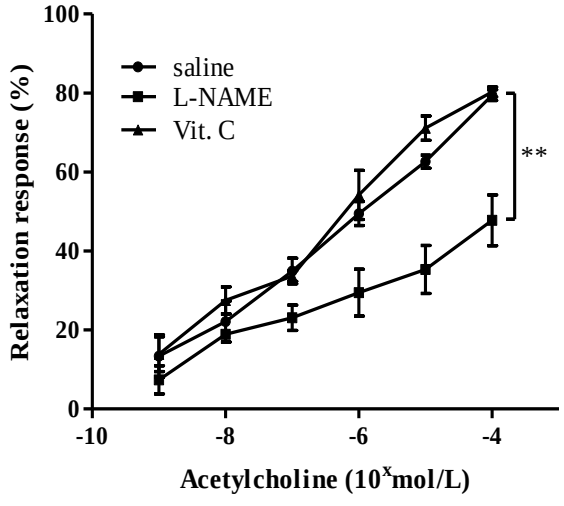


Figure 26: Endothelium-dependent relaxations elicited by Acetylcholine in mesenteric resistance arteries from Rett control (A) and curcumin treated (B) female mice without (saline) or with L-NAME or Vit C. Each point represents the mean of 6 experiments ± SEM. **P<0.01.

4.3 Vascular superoxide generation: effect of curcumin

The vascular superoxide generation has been evidenced through dihydroethidium (DHE) staining. DHE is rapidly oxidized to a fluorescent product by O_2^- produced within the cells, intercalates with DNA and stains the cell nucleus red.

In small arteries from MeCP2$^{+/-}$ mice, DHE analysis revealed a dramatic increase in superoxide anion production, as compared with WT (Figure 27). The enhanced superoxide generation was abrogated by curcumin (Figure 27).

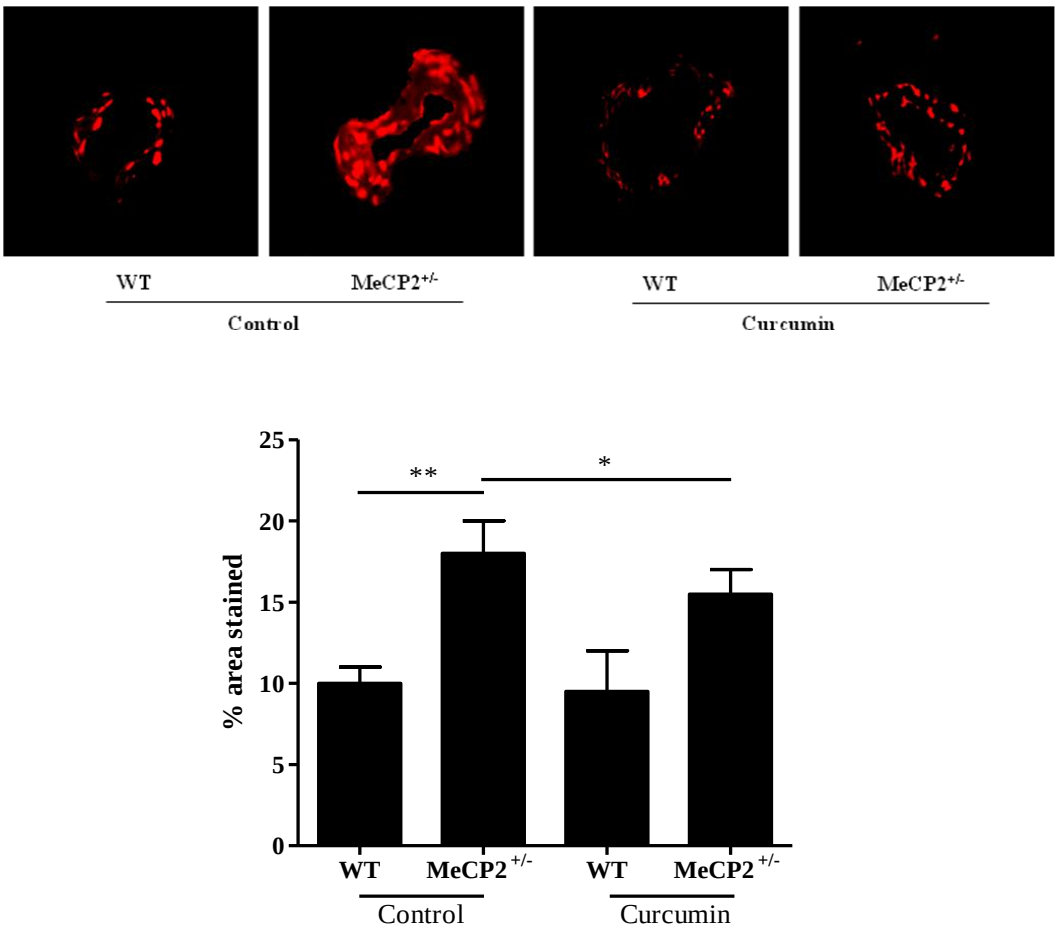


Figure 27: Representative DHE staining and quantification (bar graph) of the red signal in mesenteric arteries (magnification X 40) from WT and MeCP2$^{+/-}$ mice feed with or without curcumin. Results are given as the mean of 6 experiments ± SEM. *P<0.05, **P<0.01.

4.4 Plasma MDA levels: effect of curcumin

Malondialdehyde (MDA) is a biomarker of lipid peroxidation and an indicator of oxidative stress. We collected mice blood and separated plasma fraction by centrifugation. We tested the malondialdehyde levels in the plasma of WT, Rett and curcumin treated animals.

The comparison of malondialdehyde values measured within Rett and WT mice has shown that at baseline, Rett mice displayed high MDA levels. The statistical analysis shows a significant difference between the two groups. Curcumin administration is able to significantly reduce levels of malondialdehyde (Figure 28).

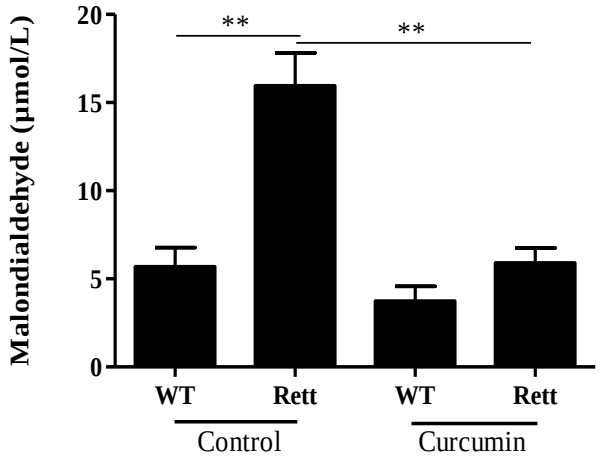


Figure 28: Oxidative stress parameter MDA. Mean±SEM Malondialdehyde (MDA) in the plasma of WT and MeCP2$^{+/-}$ mice feed with or without curcumin. **P<0.01.

4.5 eNOS expression in mesenteric vessel and aorta

The eNOS mRNA expression was measured by quantitative RT-PCR in mesenteric vessels and aorta of Rett animals, wild type age-matched littermates and curcumin treated. Consistently with the functional experiments on mesenteric vessels the Real Time-PCR provided the evidence that Rett mice have decreased levels for eNOS mRNA expression in the mesenteric arterioles and aorta (Figure 29).

The curcumin treatment restored the expression level of eNOS up to the levels of wild type animals. The same not happen in the aorta in which curcumin fails to increase levels of eNOS (Figure 29).

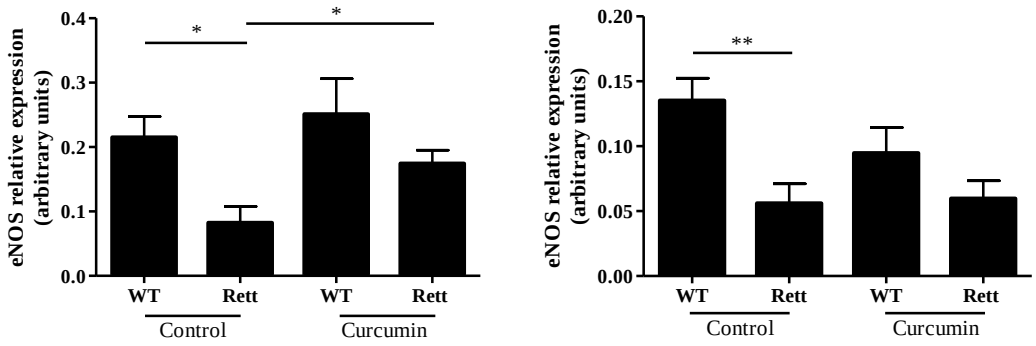


Figure 29: Quantitative RT–PCR analysis of mRNA expression for eNOS in Mesenteric vessels (A) and Aorta (B) of Wild-Type and Rett animals treated or not three weeks with 5% curcumin. eNOS has been normalized to the expression of GAPDH. Data represent the mean±SEM (*P<0.05, **P<0.01).

4.6 Vascular eNOS immunostaining

Immunostaining for eNOS protein confirms the date found in molecular analysis. High eNOS levels were constitutively and specifically present in endothelial cells of small mesenteric arterioles from WT mice (Figure 29). On the contrary, all MeCP2$^{+/-}$ animals displayed only a faint eNOS immunostaining at level of the outer vascular smooth muscle cells but no appreciable amounts of eNOS in the endothelium (Figure 30).

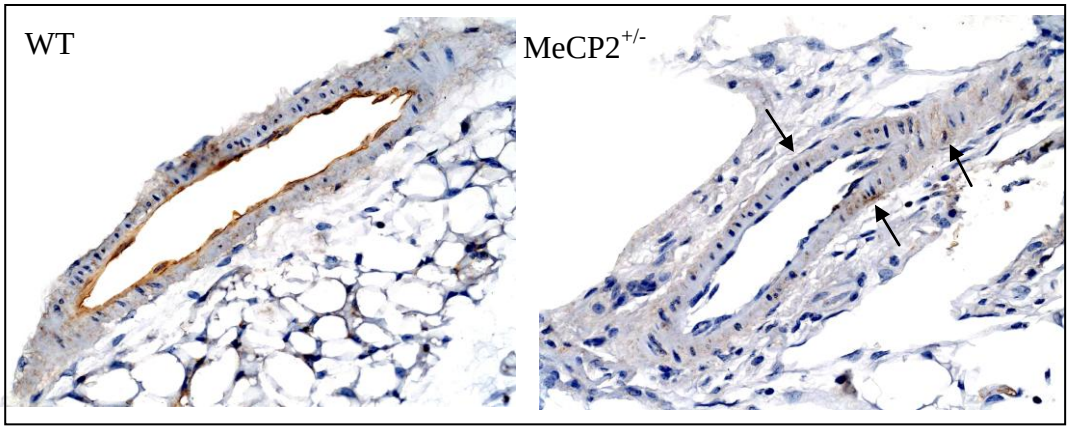


Figure 30: Representative photomicrograph of immunostaining for eNOS in small mesenteric arteries from WT and MeCP2$^{+/-}$ mice. A strong, specific eNOS staining is detected in endothelium of control vessels, while mesenteric arterioles from MeCP2$^{+/-}$ mice show only a slight eNOS immunostaining at level of the outer vascular smooth muscle cells, without any appreciable amount in the endothelial cell layer.

4.7 Body weight variation

During the 21 days of curcumin treatment the body weight of all animals was monitored. WT control and treated mice have a similar trend: they increase their weight day by day. MeCP2$^{+/-}$ have a constant weight for 14 days, then begin to fall. Instead, MECP2$^{+/-}$ curcumin treated animals increase significantly their body weight in the first two weeks and decrease dramatically in the last phase of treatment (Figure 31).

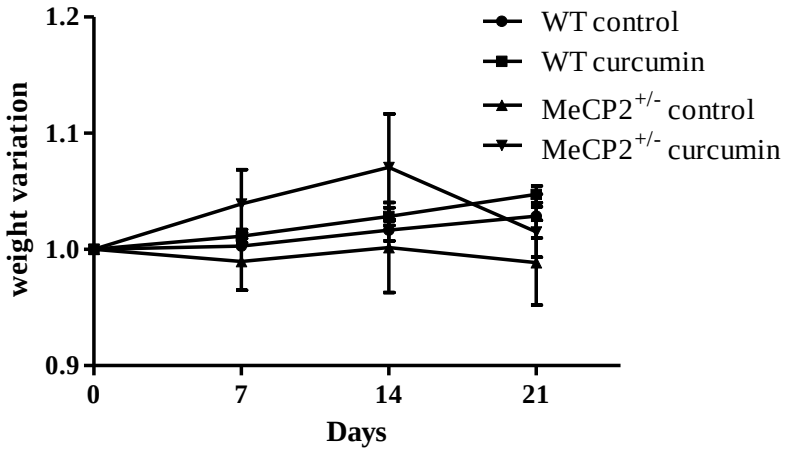


Figure 31: Curcumin effect on the animal weight, normalized at 1.0.

4.8 Rotarod test

To analyze the motor coordination learning and memory at the treatment end all animals performed the rotarod test. In this motor test the latency to fall off the rotating rod was used as an indicator of motor coordination and balance. In our experiments, no significant differences were observed between treated and untreated animals, but we can see a trend in which treated animals can best accomplish the motor task than untreated ones (Figure 32).

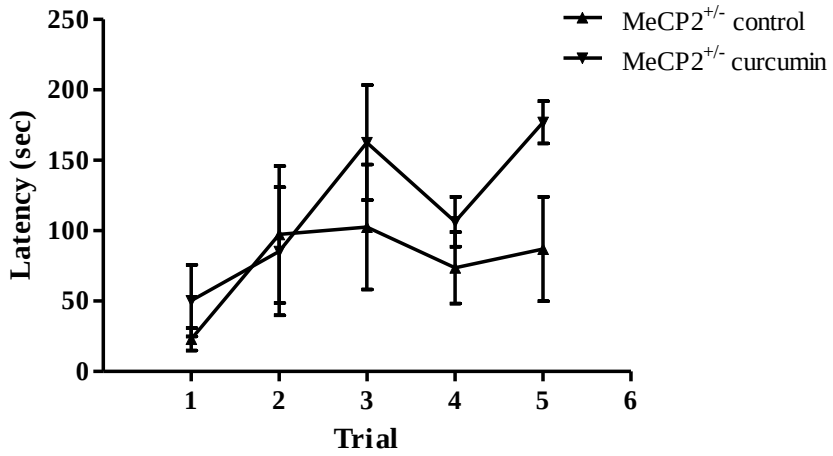


Figure 32: The averages (±SEM) for time spent on rotarod across five test trials for MeCP2$^{+/-}$ mice curcumin treated and untreated.

4.9 Stereotyped movements: effect of curcumin

Before the sacrifice, treated and untreated Rett mice and wild type age-matched littermates were recorded for two hours at three different times of the day (morning, afternoon and evening). The movies were analyzed for two behavioral parameters:

1. resting time;
2. stereotyped movements.

The observation demonstrated that MECP2$^{+/-}$ curcumin treated animal spent much less time making stereotyped movements (repeatedly and sometimes compulsively scratched the back especially with the hind limbs) than controls and spent more time to rest and sleep (Figure 33 A and B).

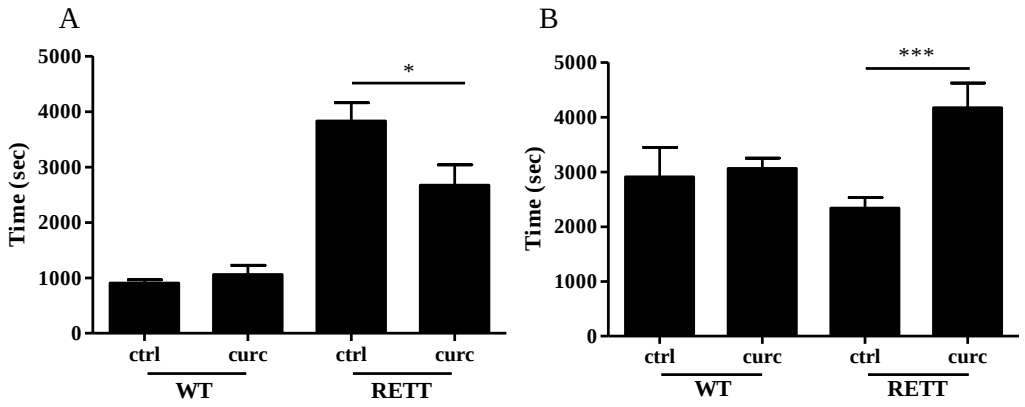


Figure 33: Behavioral observation. Percentage of time spent to perform stereotyped movements (A) and to rest or sleep (B) by Wild-Type and Rett female mice untreated (ctrl) and treated with 5% curcumin (curc). Data represent the mean±SEM (*P<0.05, ***P<0.001).

4.10 Open Field test

To assess the emotional behavior we subjected Rett and WT females to the Open field test. The test exploits the natural propensity of the animal and exploratory features such as the size of the arena, the level of lighting and background noise that can accentuate the aversion which the animal feeds to the instrument indicating how much the experimental condition has anxiety characteristics and allows the detection of many parameters such as the locomotor activity, exploratory activity, anxiety and fear. Figure 34 shows the time each animal spends at the center of the arena and near is indicated to the average for each group. Data analysis by comparing the averages of two groups, finds no significant differences in time spent in the center of the arena (Mann-Whitney test, p 0.690).

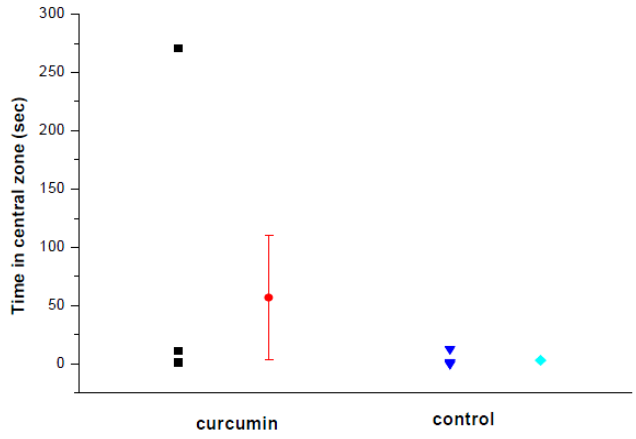


Figure 34: Time in seconds spent in the "center" of the arena for each animal in each group. In addition to individual scores shows the average for the treated group (n = 5) and controls (n = 5) with SEM.

The other parameter analyzed was the time spent in the corners of the arena (Figure 35): anxiety and fear lead the animal to stay more in the corners. Data analysis by comparing the averages of two groups, finds no significant differences in time spent in the corner of the arena (Mann-Whitney test, p 1,000).

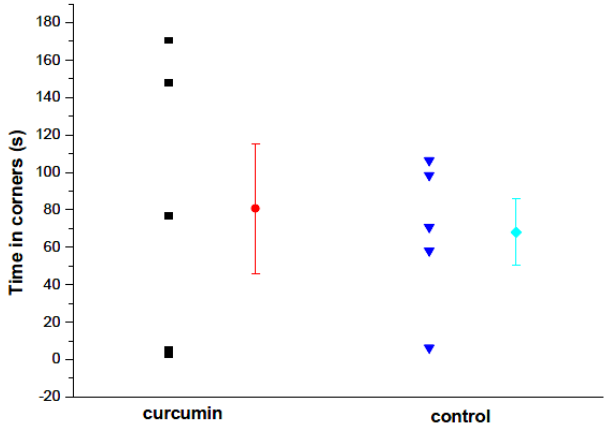


Figure 35: Time spent in the "corners" of the arena for each animal in each group. In addition to individual scores shows the average for the treated group (n = 5) and for controls (n = 5) with SEM.

The last parameter considered in this test is the percentage of time spent moving in the arena. There is no significant difference in the total time spent moving for the two groups (Mann-Whitney test, p = 0.151) (Figure 36).

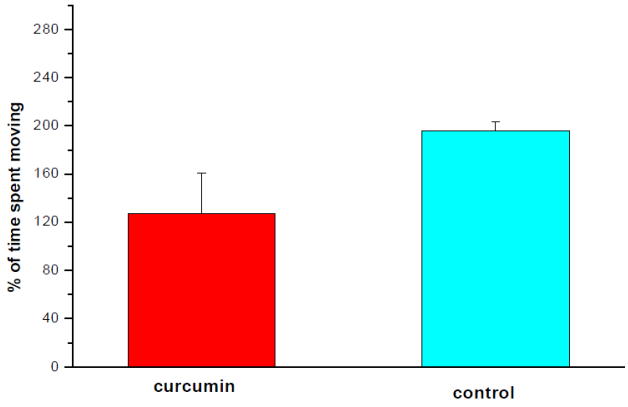


Figure 36: Percentage of time spent moving for each animal in each group. The bars indicate SEM.

All animals have explored the apparatus, probably moving in different areas, depending on the subjective appetite or not to go in the middle of it. Figure 37 shows two tracks of the run of treated and control animals.

Figure 37: Example of a path drawn by an curcumin treated animal (A) and a control one (B). The red line indicates the route taken by the animals.

DISCUSSION

Rett Syndrome is a neurodevelopmental disorder described for the first time in 1966 by the Austrian neurologist Andreas Rett. More than 40 years of research led to the discovery that RTT is caused by mutations in a specific gene: MeCP2. The discovery that RTT is a monogenic disease is particularly important to understand the physiopathology of the syndrome and to shed light on the causes of autism spectrum disorders. More than 300 associated mutations have been identified and clinical trials have been focused on drugs to enhance the quality of life for those affected. Also, scientists have made significant progress with animal studies, including effectively reversing symptoms of the disorder. The prominent CNS effects of the RTT syndrome have so far directed a major research effort into the investigation of alterations taking place in the central nervous system, almost neglecting the other somatic peripheral symptoms (Stearns et al, 2007). Some authors investigated the cardiovascular functions in human patients and in RTT animal models, given that they believe that the 26% of sudden deaths may be due to abnormalities in the autonomic regulation of hearth rate (Bissonnette et al, 2007). Moreover the frequent peripheral circulation problems of RTT patients are poorly investigated. Only in 1997 Bjure and colleagues described abnormalities in the blood flow in the brain of RTT girls (Bjure et al, 1997).

In our studies we investigated for the first time the peripheral microcirculation in a symptomatic MeCP2 knockout mouse model to understand the causes of vascular dysfunction and to suggest an effective therapeutic treatment able to counteract the peripheral symptoms.

In the first set of experiments, to investigate the function of the peripheral microcirculation in the symptomatic MeCP2 knockout mouse, we used the

pressurized myograph system to study the mesenteric arterioles vascular reactivity in wild type animals and in young MeCP2$^{y/-}$ male and in adult MeCP2$^{+/-}$ female mice. Subsequently we evaluated the impact of curcumin administration on vascular function and on properly chosen behaviuoral parameters in our female RTT mouse model.

The first major finding of our study concerns the demonstration of endothelial dysfunction and the major mechanisms responsible for this alteration in RTT male and female animal model. In the mesenteric arterioles of the MeCP2$^{+/-}$ mice, we observed a reduction in the vascular response to acetylcholine, an endothelium-dependent vasodilator, together with a preserved relaxation to sodium nitroprusside, a direct smooth muscle cell relaxant compound, thus demonstrating the presence of endothelial dysfunction in such animal model of disease. To assess NO availability and oxidative stress, we used L-NAME and ascorbic acid, respectively. In MECP2$^{+/-}$ mice, the inhibitory effect exerted by L-NAME on acetylcholine responses was significantly lower compared with that observed in controls, indicating reduced NO availability. Ascorbic acid improved the response to acetylcholine, suggesting that ROS generation contributes to endothelial dysfunction which characterizes such disease. Taken together, these findings indicate that ROS generation represents a major mechanism accounting for endothelial dysfunction in peripheral microcirculation of MeCP2$^{+/-}$ mice, resulting in NO breakdown.

The results are more dramatic in mesenteric vessels of MeCP2$^{y/-}$ in which we evidenced that the NO availability is almost absent: the relaxation in the response to acetylcholine was totally refractory to L-NAME, while ascorbic acid only

slightly restored the inhibitory effect of L-NAME. This suggests a quite irreversible exhaustion of the L-arginine-NO pathway. In these animals also emerged a significantly reduced relaxation to sodium nitroprusside, demonstrating that the deletion of MeCP2 alters the endothelium functionality but involves also the underlying smooth muscle cells.

In line with these functional data, $MeCP2^{+/-}$ mice showed an increased intravascular superoxide anion generation and displayed higher plasma values of MDA, a systemic marker of oxidative stress. Our results in rodent agree with data in humans reporting the presence of systemic oxidative stress in RTT patients, in particular Sierra and De Felice reported elevated plasma levels of oxidative stress, decreased superoxide dismutase activity, and increased markers of lipid peroxidation. This is an intriguing and long debated aspect in Rett syndrome pathogenesis (De Felice et al, 2009; Sierra et al, 2001). Moreover the central nervous system is particularly sensitive to the adverse effects of oxidative stress as demonstrated in several nervous system pathologies (Atmaca et al, 2004).

The major role of ROS in determining NO-related endothelial dysfunction in $MECP2^{+/-}$ mice is in strict relationship with the second novel finding of our study, which deals with the vascular beneficial effect of curcumin. Indeed, vessels from curcumin treated $MECP2^{+/-}$ mice showed an increased relaxation to acetylcholine, which became sensitive to L-NAME, while ascorbic acid was no longer able to affect the response to acetylcholine.

The discovery that with curcumin L-NAME can inhibit relaxation to acetylcholine, an effect not exerted under baseline conditions, implies that NO availability is restored after pharmacological treatment. It is worth of noting that,

after curcumin administration, the restored inhibiting effect of L-NAME on acetylcholine was similar to what observed by ascorbic acid at baseline. Therefore, these findings suggest that the mechanism responsible for the beneficial effect of curcumin is probably specific and related to its antioxidant properties. This possibility is strengthened by the finding that curcumin also prevented the intravascular superoxide excess, and normalized the increased plasma levels of MDA, which resulted no longer different from controls.

Our findings extend to the peripheral microcirculation of $MECP2^{+/-}$ mice previous evidence showing the beneficial antioxidant properties of curcumin on other animal models affected by oxidative damage (Majithiya & Balaraman, 2005; Sompamit et al, 2009).

Any way in the presence of ascorbic acid, either the relaxing effect of acetylcholine or the inhibitory effect of L-NAME on acetylcholine, although significantly improved, but not reach normal values. Thus, we cannot exclude that other ROS-independent mechanisms may contribute to endothelial dysfunction in MeCP2 knockout mice.

Rett syndrome and its symptomatology are intimately connected to the uncorrected interpretation of chromatin epigenetic modification (acetylation-methylation) caused by mutated or abnormal expression of MeCP2 (Agarwal et al, 2011). Intriguingly, several reports indicated the chromatin structure and the epigenetic modifications as key determinants for eNOS expression. MeCP2 protein has in fact been repeatedly localized bound to eNOS promoter and chromatin structure as well as chromatin modification (methylation and histone acetylation) are critical to the cell-specific expression of eNOS (Chan et al, 2004).

This context is consistent with MeCP2 playing an upstream action on eNOS expression and consequently on the levels of NO. In fact what we observed in histochemical assay from mesenteric vessels of MeCP2+/- mice was an abnormal expression of eNOS peptide in vascular smooth muscle cells, and any expression in endothelial cells. This lasting finding requires further experiments to be explained, but we can suppose the involvement of MeCP2 in the regulation of expression of eNOS. In line with the reduced NO availability herein reported, we found decreased eNOS mRNA expression in mesenteric vessels and in aorta of the MeCP2 deficient mice. We confirmed the reduction of eNOS expression also at protein level, indeed the immunostaining on isolated mesenteric vessel demonstrated that in the $MeCP2^{+/-}$ animals eNOS protein is totally absent in endothelium respect with WT littermates in which the protein is specifically expressed in this vascular compartment. There is the possibility that the *MECP2* mutation leads to an incomplete eNOS expression, with a consequent reduced production and release of NO. This alteration might contribute to the reduced NO availability occurring in our $MeCP2^{+/-}$ female mice, independently of increased ROS generation. Another possibility, not necessarily alternative, highlights eNOS as a putative intravascular source of ROS. This because the low eNOS expression might result by a deficiency of the substrate arginine or the cofactor tetrahydrobiopterin. This process, called "NOS uncoupling", implies that the physiological activity of the enzyme for NO production is decreased and switched to the NOS-dependent ROS generation (Verhaar et al, 2004). To substantiate this conjecture, we documented a normalization of eNOS mRNA expression in mesenteric vessels but not in aorta from curcumin-treated MeCP2 animals, thus

suggesting that in a condition of ROS scavenging, such as that occurring under curcumin therapy, eNOS is switched back to its physiological function to generate NO in mesenteric district but not in regions distant from the absorption site. We recognize these as speculative hypothesis which needs future confirmations.

Our findings may have clinical relevance. It is widely recognized that in physiological conditions, vascular NO not only causes vasodilation, but also protects the vessel wall against the development of atherosclerosis (Davignon & Ganz, 2004). In contrast, the impaired NO activity and the enhanced activity of vasoconstrictors, including ROS, are responsible for impaired vasodilator capacity. The imbalance of this equilibrium towards oxidative stress, beyond the altered vasomotricity, also initiates a variety of active processes, such as inflammation, cell migration and proliferation, relevant for the endothelial surface and adjacent cells. For these reasons, the altered NO-mediated endothelial function and the increased ROS generation which characterize the peripheral microcirculation of MeCP2 knockout animals, might have a causal relationship with the poor circulation, present in the extremities of RTT patients, including cold and cyanotic legs.

The present study suggests curcumin as a promising medication to treat some symptoms of the Rett syndrome. The implementation of curcumin as a therapy to improve vascular system-related symptoms of the RTT syndrome needs to face some drawbacks that are outlined below. The main disadvantages of curcumin oral administration is its poor bioavailability, as it is principally accumulated in gastrointestinal tissues and metabolized in the liver Garcea and colleagues demonstrated that following the oral administration of 3.6 g/d curcumin for seven

days in colorectal cancer patients, curcumin is detectable in peripheral circulation (Garcea et al, 2005). In our system the proximity of the mesenteric vascular system to the intestine could be the reason of the striking action of curcumin, and indeed represents a proof of principle of its action on RTT mice vessels. Further studies are needed to optimize curcumin administration method.

The adult MeCP2$^{tm1/Jae}$ animal similarly to RTT patient develops typical stereotyped movements (Stearns et al, 2007). This behavioral aspect to date without any effective pharmacological therapy is often injurious and the girls are forced to wear gloves to avoid severe self-damage. Oxidative stress and stereotypic movements association is indeed well supported by a growing body of evidence in different animal models and for example in patients affected by obsessive compulsive disorders (Ghanizadeh, 2011; Guldenpfennig et al, 2011; Izzo et al, 2005). Specifically Bishnoi and colleagues assayed in rat animal model the benefical antioxidant effect of curcumin on central nervous system and on stereotyped movements (Bishnoi et al, 2008). In this scenario the curcumin benefical effect assayed by movie recording is not surprising. Our results on behavioural tests reported that curcumin administration is able to improve stereotyped movements and resting time but we not evidenced significant changes in emotional behavior and only an improvement trend of motor deficit. The increase of body weight in the first two weeks of treatment indicates that curcumin counteracts the RTT patients gastrointestinal problems that lead to the underweight tendency.

In conclusion, the present study indicates that mesenteric vessels from MeCP2$^{+/-}$ female mice are characterized by an altered endothelium-dependent relaxation

caused by a reduced NO availability due to an increased ROS generation, and by a lower endothelial eNOS expression. These alterations, as well as the pathological stereotyped movements that characterize pathological animals, were reversed by curcumin administration. These data revealed that the RTT animal model shares some of the components of the clinical oxidative and vascular impairment of RTT patients, who often present circulatory problems that become increasingly severe in adulthood. It is proposed that the peripheral vascular system of these animals may be a source of excessive free radicals contributing to the local and systemic oxidative stress, and that curcumin based therapy may at least partly improve RTT symptoms derived from these redox alterations.

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