Behavioural phenotyping of genetic mouse models of neuropsychiatric disorders: The case of Rett syndrome

Bianca De Filippis - bianca.defilippis@iss.it
Sect. Behavioural Neuroscience, Dept. Cell Biology & Neuroscience, Istituto Superiore di Sanità

Abstract

The creation of mouse models with genetic aberrations characteristic of human clinical diseases has provided a major breakthrough in the understanding of neuropsychiatric disorders. The study of behaviour, which is the ultimate output of brain, plays a crucial role in this context: behavioural phenotyping of genetically modified mice provides functional information hardly detectable using neurobiological evaluations alone. Rett syndrome (RTT) is a pervasive developmental disorder, primarily affecting girls. RTT causes a wide variety of debilitating symptoms and no cure currently exists. Mutations in the gene encoding methyl–CpG–binding protein 2 (MeCP2) have been found to be responsible for about 90% of classical RTT cases. After the discovery of a monogenic origin for RTT, several lines of mice carrying endogenous Mecp2 mutations have been generated. In particular, we focussed on a mouse model which expresses a truncated form of the *Mecp2* gene (Mecp2-308) and displays a milder phenotype (e.g. longer lifespan), thus allowing longitudinal fine-grain neurobehavioural analysis. The detailed characterization of the behavioural responses of Mecp2-308 mice across the lifespan we carried out (starting from soon after birth till adulthood) evidenced several alterations in the normal range of strain-specific behaviours (e.g. increased anxiety-like behaviours, impairments in nest building abilities) and an interesting timeline in the progression of symptoms. Challenges with psychoactive drugs were also adopted to assess the functional integrity of selected neurochemical systems potentially targeted by Mecp2 mutations. Particular attention was devoted to the early phases of development, the so-called pre-symptomatic phase: by means of experimental protocols that take into account the practical constraints imposed by the peculiar physiological and behavioural responses of an immature subject, deviations from the age-dependent onset of normal response patterns were also highlighted in Mecp2-308 mice. In particular, a significant decrease in ultrasound vocalizations emissions, increased arousal and hyperactivity and reduced motor coordination were evidenced during the first postnatal days in this RTT mouse model. To complement our behavioural analyses, neurobiological evaluations were performed in fully symptomatic Mecp2-308 mice which provided evidence, for the first time in a RTT mouse model, of signs of atrophy in astrocytic cell populations and impairments in the central cholinergic function.

1. Mouse models of neuropsychiatric disorders

Research in rodents has played a central role in understanding the neurobiological bases of several pathological conditions and how they can be treated. Recently, the development and application of novel molecular technologies, such as *gene targeting* procedures, has led to a sudden increase in the use of mice in neuropsychiatric research as in other biomedical disciplines. In fact, the introduction into pre-determined sites in the genome of planned mutations (null mutations as well as more subtle changes which alter, but do not eliminate gene function) can be engineered in the rat and even in higher mammals. The mouse, however, is uniquely suitable to these techniques (Cryan and Holmes 2005). These advances in the field of genetics, have led to the creation of mouse models with genetic aberrations characteristic of human clinical disorders (Tecott 2003). Transgenic mice have been developed for many neurotransmitters, receptors, second messengers, transporters, and transcription factors.

Since behaviour is the ultimate output of brain, behavioural phenotyping of genetically modified mouse models of neuropsychiatric disorders provides functional information that cannot be detected using molecular, cellular or histological evaluations. Such functional information help identifying the role of specific genes in neuropathologies, but it also provides a framework for understanding the role of genes in behaviour, identifying key stages of human brain development, and, eventually, targets for potential therapeutic interventions. To unravel the effects of genetic manipulations, deviations from the normal range of strain-specific behaviours and the age-dependent onset of normal response patterns can be investigated. Another behavioural phenotyping strategy can be based upon the study of selected brain regions and of those neurochemical systems specifically targeted by genetic alterations: to assess their functionality, behavioural tasks known to be controlled by those circuits could represent a powerful tool and a very sensitive assay. Furthermore, the analysis of deviations in response to challenges with psychoactive drugs (direct receptor agonists or antagonists, acting on specific neurotransmitter systems) can complement this strategy (Bignami 1996). The use of drug challenges may indeed unmask neurobehavioural alterations not detectable under baseline testing conditions and provide crucial information on neurobiological impairments that can be subsequently confirmed *in vitro*.

2. Validity of mouse models

It goes without saying that we can never fully recapitulate human neuropsychiatric symptomatology in the mouse. Given the substantial differences in the anatomy of the brain between humans and mice, particularly the cerebral cortex, some human neuropsychiatric symptoms (mainly those related to the ability of elaborating complex psychological concepts such as low self-esteem) are impossible to model in mice. Nonetheless, the cerebral cor-

tex is closely interconnected with sub cortical structures that are well conserved and show marked similarities across mammals (e.g. a common structural organization, consisting of the cerebral hemispheres, diencephalon, midbrain, cerebellum, pons and medulla) (Tecott 2003; Jones 2002). In addition, there are many physiological and behavioural responses that have been evolutionarily conserved between mammalian species. Therefore, to understand human behaviour and disease, and to elucidate the neural circuits and genetic factors sub serving them, it is possible to study such responses in rodents. With this principle in mind, three criteria for evaluating whether an animal model can be considered as a good model of a neuropsychiatric disorder have been proposed (McKinney 1984).

Construct validity implies a theoretical analogy to the cause of the human disease. Mutant mice with a mutation in a gene implicated in a neuropsychiatric disorder have construct validity for that inactivation or polymorphism of the human gene. Neuroanatomical lesions, prenatal drug exposures, and environmental toxins offer other examples of causes of human diseases that can be modelled in mice. *Face validity* implies a theoretical analogy to the symptoms of the human disease. Behavioural symptoms, neuroanatomical, neurophysiological and neurochemical abnormalities are examples of disease components that can be modelled in animals. *Predictive validity* implies specificity of responses to treatments that are effective in the human disease. A specific class of drugs that ameliorates the human symptoms should reverse the traits in the animal model. Classes of drugs that are ineffective in the human syndrome must similarly be ineffective in the animal model.

The more similarities in *construct, face,* and *predictive validity* between an animal model and the human disease, the stronger the model, and the more useful it will be for elucidating the contribution of specific gene alterations and gene-environment interactions to the phenotype of neuropsychiatric disorders and for translational evaluation of pharmacological, behavioural, and other treatments for the disease.

3. Rett syndrome

Rett syndrome (RTT) is a pervasive developmental disorder, primarily affecting girls with a prevalence of 1 in every 10,000 births. Although up to five variants have been identified so far, three quarters of the cases meet the diagnostic criteria for classic RTT (Hagberg 2002).

Mutations in the gene encoding methyl–CpG–binding protein 2 (Mecp2) have been identified as clear etiological factors in about 90% of classical RTT cases (Amir et al. 1999; Chahrour et al. 2007). The Mecp2 gene encodes two closely related proteins which selectively bind to methylated CpGs, sequences of the DNA constituted by the repetition of the same dinucleotide (a cytosine followed by guanine) and mostly located within the promoters of genes (Jones et al. 1998). Although it is clear that Mecp2 plays multifunctional roles at the cellular level, primarily acting as a transcriptional repressor, the exact nature of these roles is currently unknown (Chahrour et al. 2008; Ogier et al. 2008). Moreover, although several Mecp2-target genes have been proposed [for an overview: (Chadwick et al. 2007)], the mechanisms leading to the severe, progressive and specific neuronal dysfunctions when these genes are mutated remain to be elucidated. Importantly, an X-linked dominant mode of inheritance characterizes Mecp2 gene and accounts for the skewed sex ratio observable among RTT patients: RTT mainly affects females, in addition to the fact that most of the hemizygous males and homozygous females do not survive (Percy et al. 2005).

RTT patients undergo an apparently normal prenatal and perinatal development until about 6-18 months of age. This early phase is followed by a regression period, characterized by both a profound loss of acquired developmental skills in the areas of social contact, communication and hand use and a deceleration of head growth, usually leading to microcephaly (Hagberg 2002).

At the end of this period, development reaches a plateau associated with a wide variety of RTT peculiar symptoms. These include stereotyped hand movements, major breathing abnormalities, bloating, EEG irregularities, sleep problems, gait dispraxia, back deformities, feeding abnormalities as well as autistic-like behaviours [for a detailed review of symptoms see: (Mount et al. 2001; Hagberg 2002)]. During the last part of their life, RTT patients undergo a noteworthy worsening of motor performance. Lifespan is extremely variable and some individuals survive up to 70 years of age (Chahrour et al. 2007).

Despite an homogeneous genetic origin, both the severity of symptoms and the progression of the disease of the patients carrying a mutation in the Mecp2 gene can be extremely variable (Erlandson et al. 2005). Different causes have been proposed to contribute to the great phenotypic variability among RTT patients. First of all, different mutations have been described to determine different phenotypes. For instance, milder phenotypes have been found to be associated with C-terminal deletions, a type of mutation which accounts for about 10%of RTT cases, whereas early truncating mutations are responsible for more severe symptoms (Chahrour et al. 2007).

Moreover, in heterozygous females X-linked genes are subjected to the X-chromosome inactivation phenomenon, where one of the two X chromosomes is randomly inactivated in every cell of the body. The RTT phenotype has been observed to vary depending on the number of cells expressing the wild type allele versus the mutated one. A skewed pattern favouring the wild type allele, would result in a milder phenotype (Hoffbuhr et al. 2002).

No cure currently exists for treating this devastating disorder. Only interventional modalities aimed at improving quality of life of RTT patients are available at the moment. These include rehabilitation interventions for the improvement of physical impairments (Lotan 2007) as well as alternative symptomatic interventions, ranging from pet therapy to acupuncture (Lotan 2007).

4. RTT mouse models carrying Mecp2 mutations

Two decades ago, "gene targeting" procedures were developed for the introduction into pre-determined sites in the mouse genome of planned mutations (Tecott 2003). As in RTT Mecp2 gene mutations have been found to be responsible for about 90% of classical RTT cases, strategies employing gene targeting have been used to generate several lines of mice carrying endogenous Mecp2 mutations.

These mutant mice are regarded as good models of RTT for their high construct validity [i.e. the extent to which a model reproduces the etiology and pathophysiology of a disorder (McKinney 1984)]. Moreover, indications are available suggesting a high face validity for this models [i.e. the degree to which a model resembles the symptoms of a disorder (McKinney 1984)], as Mecp2 mutant mice have been reported to recapitulate many RTT symptoms (see below).

RTT mainly affects girls, due to the localization of Mecp2 gene on the X chromosome; most of the mouse studies, however, focused on phenotype of hemizygous male mice. This apparent discrepancy is primarily because of the unpredictability of heterozygous female phenotypes, deriving from the X-chromosome inactivation phenomena. As a matter of fact, the severity of symptoms is extremely variable in heterozygous females, depending on the number of cells expressing the wild type versus the mutated alleles: the higher the ratio, the less severe the RTT phenotype. In addition, this variability seems to be established by chance. As a consequence, hemizygous male mice are at the moment considered the most suitable model to investigate the role of Mecp2 gene mutations on the development of RTT-like symptoms, as they allow the elimination of confounding effects of a variable phenotype. Innovative models should take into account the need to explore RTT phenotype within the constraints and/or possibility provided by a more appropriate female-hormonal milieu.

The first RTT mouse models were described in the same issue of 2001 in Nature Genetics from two different laboratories (Chen et al. 2001; Guy et al. 2001). Guy and colleagues (Guy et al. 2001) generated Mecp2 null mice lacking exon 3 and 4 (hemizygous males), thus blocking Mecp2 expression in the entire organism (Mecp2Bird); Chen and colleagues (2001) compared i) a full knockout generated by the targeting deletion of exon 3 (Mecp2Jae), ii) a conditional knockout carrying a Nestin-Cre transgene blocking embryonic Mecp2 expression selectively in the brain (CNS knockout) and iii) a conditional knockout generated by the introduction of a CamK-Cre93 transgene blocking Mecp2 expression in the postmitotic neurons in the forebrain (forebrain knockout). Phenotypic differences among the three different lines were surprisingly limited, suggesting that most of the traits were due to the loss of Mecp2 gene function in the brain.

Subsequently, a RTT mouse model was described by Zoghbi's group, which expresses a truncated form of Mecp2 (Mecp2-308), a mutation commonly found in RTT patients (Chen et al. 2001; Shahbazian et al. 2002).

More recently, a new RTT mouse model in which Mecp2 is removed from Sim1-expressing neurons in the hypothalamus has been generated using Cre-loxP technology (hypothalamus knockout), in order to uncover endogenous functions of Mecp2 in the hypothalamus (Fyffe et al. 2008). Other new and interesting RTT mouse models include: 1) a selective basolateral amygdala loss of Mecp2 in adult mice (basolateral amygdala knockout) has been examined (Adachi et al. 2009); 2) Mecp2-null mice, created by targeted deletion of the methyl-binding domain (MBD) of the Mecp2 gene (Mecp2Tam) (Pelka et al. 2006); 3) a mouse model carrying a different (i.e. shorter) truncated form of Mecp2 gene (Mecp2-168) (Lawson-Yuen et al. 2007).

Interestingly, Mecp2 overexpression also produces RTT-like symptomatology in different transgenic lines (Collins et al. 2004). This observation underlies the importance of a precisely regulated Mecp2 gene expression. In particular, different levels of Mecp2 gene expression have been observed to correlate with the severity of the RTT-like phenotype: the higher Mecp2 gene expression, the earlier the onset of symptoms as well as the shorter the life span. Despite a great variability in the progression of symptoms, all the different transgenic lines display characteristic hind-limb clasping, kyphosis, aggressiveness, and, to some degree, a premature death. Moreover, the transgenic line expressing Mecp2 at 2-fold wild type levels (Mecp2-Tg1) also shows seizures, EEG abnormalities, decreased anxiety-like behaviors, hypoactivity, and suprisingly, enhanced motor skills and hippocampal learning, as well as increased synaptic plasticity.

5. Onset of symptoms, life span and general health in RTT mouse models

Different developmental phases can be identified in the RTT mouse models described so far as well as in RTT patients. All RTT patients experience an early developmental phase where no obvious deficits (i.e. visible by gross examinations) can be detected. After the onset of symptoms, RTT subjects undergo a progressive worsening until premature death.

In the Mecp2Bird model (Guy et al. 2001), male and female mice appeared normal till the third postnatal week, afterwards both sexes developed gross abnormalities (with sex-dimorphic time course) consisting of a stiff, uncoordinated gait and reduced spontaneous movements. Most animals subsequently developed hind-limb clasping and irregular breathing. Uneven wearing of the teeth and misalignment of the jaws was also recurrent. Testes of Mecp2-null mice were always internal. A distinct feature of the phenotype was varying body weight, which was dependent on genetic background (C57Bl/6 gave rise to underweight animals, whereas, after crossing to a 129 strain, F1 animals became heavier than siblings since they were 8 weeks of age). In Mecp2Bird hemizygous males, variable progression of pathology occurred between the fourth and seventh postnatal week leading to rapid weight loss and death (around eighth postnatal week). By contrast,

females initially showed no symptoms and raised normal litters, and only after the twelfth postnatal week, acquired inertia and hind-limb clasping phenotypes. More pervasive unambiguous symptoms (e.g. irregular breathing) are present only in one half of the heterozygous females by nine months.

Also in the Mecp2Jae model, onset of symptoms has been extensively described (Stearns et al. 2007). As early as 4 weeks of age mutant male mice could be occasionally identified by an altered gait. A significant loss of body weight, body tremors and shaking paws were evident by 5 weeks of age, whereas piloerection and periods of labored breathing were noted as early as 6 weeks of age. Heterozygous mutant females, although their body weight was slightly reduced compared to wild type (WT) females by 5 weeks of age, seemed normal for the first four months and began to show symptoms such as reduced activity and ataxic gait at a later stage. In association with the appearance of symptoms, females were also reported to gain weight. Piloerection, hind-limb clasping and heavy breathing took six months or longer to develop.

Mice with a truncated mutation (Mecp2-308) similarly recapitulate many RTT features (Shahbazian et al. 2002). Mutant male mice exhibited no apparent abnormalities until around 6 weeks of age, when tremors were detectable while suspending mice by the tail. At 4 months of age, tremors were apparent by visual observation alone. After 5 months of age, forty percent of animals developed kyphosis; after 8 months of age, the fur of mutant mice was noticeably more oily and disheveled than that of wild types. Spontaneous behavioural myoclonic jerks and seizures were observed in several mutant mice. Body weight was within the normal range, and mice were fertile. Truncated Mecp2-308 male mice were anecdotally reported to rapidly and repeatedly move their fore-limbs, often bringing them together and sometimes holding them together for several seconds when they were undisturbed in their home-cage or suspended by the tail. Moreover, as early as 6 weeks of age mutant male mice are reported to develop periocular inflammation and bleeding usually accompanied by bacterial infections. In a mixed background (129SvEv x C57Bl/6) (Young et al. 2004), and ataxia and breathing abnormalities appear (Moretti et al. 2005). Most Mecp2-308 male mice survived at least 1 year of age, whereas heterozygous female mice displayed milder and variable features starting from 1 year of age.

Although their characterization is quite far from complete, also the other RTT models have been reported to recapitulate RTT symptoms. In the forebrain knockout mice, for instance, no obvious initial phenotypic differences were noted between hemizygous mice and sex-matched controls. At 16 weeks of age, however, mutant mice became heavier than WT and started showing hind-limb clasping. In addition, weight gain went on increasing as they became older (Gemelli et al. 2006).

In another Mecp2-null model, the Mecp2Tam, depending on the background, hemizygous male mice have been reported to survive up to 20 weeks of age. Unusual gait, hind-limb clasping, dishevelled fur, laboured breathing, tremors and seizures were observed in this mouse model. At about 5 weeks of age, hemizygous male mice start failing to thrive. No differences between heterozygous and WT females were evident until 13 weeks of age (Pelka et al. 2006).

A longer lifespan has been observed in the truncated Mecp2-168 model. Hemizygous male mice have been reported to survive until about 12 weeks of age and to show, by 7 weeks of age, significant hind-limb atrophy and clasping, hypoactivity, and breathing irregularities (Lawson-Yuen et al. 2007). Interestingly, fore-limb stereotypes have been reported for this mouse model as well as for the Mecp2-308 (Lawson-Yuen et al. 2007). Heterozygous females showed significant symptoms (i.e. hind-limb clasping and breathing irregularities) by approximately 6 months and survived more than 1 year.

Hypothalamus knockout mice have been reported to start showing symptoms at about 7 weeks of age, when both stereotypies and kyphosis appeared (Fyffe et al. 2008). However, no reduced lifespan has been observed in this RTT mouse model. Interestingly, loss of Mecp2 in Sim1-expressing neurons resulted in mice that recapitulated the abnormal physiological stress response that is seen when Mecp2 is absent in the entire brain. Quite surprisingly, this Mecp2 conditional knockout mice were reported to be aggressive, hyperphagic, and obese (Fyffe et al. 2008).

As a whole, age at onset of symptoms varied largely in those models, with earliest onset in Mecp2Bird male mice (3 weeks), and latest in truncated Mecp2-308 hemizygous mice. Same profile is evident in life span with Mecp2Bird mice living till the 8th postnatal week.

As mouse models are expected to be enormously beneficial for determining the functional outcome and the effects of gene mutations on organic and cellular functions, a complete and accurate phenotyping of these mutant mice could offer a great opportunity to RTT research. In addition, a robust rodent model can have translational value in offering preclinical surrogate markers to evaluate treatment efficacy (Crawley 2007).

6. The neurobehavioural phenotype of Mecp2-308 mice

Whereas Mecp2-null mice are the most studied for altered neuronal morphology and network, Mecp2-308 mice (Shahbazian et al. 2002) are advantageous for behavioral studies due to the delayed onset of symptoms (4 months of age) and prolonged life-span (12-15 months) (Ricceri et al. 2008). Exploiting this feature, we carried out a study aimed at better characterizing the neurobehavioural phenotype of Mecp2 mutant mice.

First step in the analysis of Mecp2-308 mice was therefore to gather a set of data that are missing so far: a detailed behavioural phenotyping during that early developmental phase where no obvious deficits can be detected. As a matter of fact, as well as defining an Alzheimer animal model via its behavioural characterization only in the preweaning phase could be at least considered hazardous, it

is similarly limiting and inappropriate to describe adult, but not infant behaviour in animal models of neurological disorders with early onset and developmental pathology.

A number of tests and experimental protocols are now available that take into account the practical constraints imposed by the peculiar physiological and behavioural responses of an immature subject (Spear 1990; Branchi et al. 2002). Keeping in mind Pat Bateson's cardinal view of neurobehavioural development in mammals, as a process akin to the metamorphosis of a caterpillar into a butterfly, we can investigate appropriate behavioural endpoints for each selected maturational step, and use standardized methodological procedures to assess sensory-motor, emotional and cognitive domains in developing mice. Time of onset of selected somatic changes and the time of first appearance of various reflexes and behavioural patterns show a remarkable regularity, providing an effective tool to assess possible neurobehavioural/developmental alterations.

In Mecp2-308 mutant male mice, a picture of impaired emotional communicative behaviour (a significant decrease in ultrasound vocalizations emissions) as well as increased arousal and hyperactivity and reduced motor coordination were evidenced during the first postnatal days, the so-called pre-symptomatic phase (see (De Filippis et al. 2010)). Our data provided evidence of precocious behavioural markers of RTT to be exploited as early diagnostic tools as well as to test the efficacy of early intervention, when recovery could be more likely. Given the strict interplay between genes and environment during the development of a healthy individual, the possibility of an early intervention can result particularly important for RTT patients to reduce most of the carry-over consequences of a deviant developmental trajectory.

The behavioural characterization of Mecp2-308 mice also provided an interesting timeline of the progression of symptoms in this RTT mouse model: increased anxiety-like behaviours, in the absence of changes in cognitive performance and motor coordination, were found at an early stage of the disease (2 months of age, (De Filippis et al. 2010)). These results followed the observation that increased anxiety-like behaviours in fully symptomatic Mecp2-308 mice (4-6 months of age) are accompanied by an abnormal stress response (as evidenced by elevated serum corticosterone levels) and an increase in the paraventricular nucleus of the hypothalamus (PVN), the central amygdala, and the bed nucleus of the stria terminalis, in the expression of the Crh gene, which encodes for the neuropeptide Corticotropin-releasing hormone (CRH) (McGill et al. 2006). CHR plays a crucial role in the coordination of the behavioural and physiologic response to stress (Holsboer et al. 2010). Apart from mediating the acute responses to stress, CRH also mediates the long-term impact of stress on the brain: it potently modulates neuronal morphology by activating Rac1, a member of the Rho family of GTPases that regulates the actin and microtubule cytoskeleton (Swinny et al. 2006). In line with this observation, the constitutive activation of RhoGTPases by CNF1 inoculation in Mecp2-308 mice did not improve the anxiety profile in this RTT mouse model (see (De Filippis et al. 2012). Given the identification of Crh as a target of Mecp2, it is not surprising that, as evidenced in our study, anxiety-like behaviours are a sound feature of RTT and alterations in this behavioural domain are the first to appear during the symptomatic phase in Mecp2-308 mice.

Interestingly, in a recent report, long-term demethylation of the Crh gene and a significant increase in Crh mRNA levels in the PVN were found in adult mice after ten consecutive days of social defeat, a protocol which is known to induce anhedonia and social avoidance (Elliott et al. 2010). Because of the ability of Mecp2 to read the methylation status of the promoter of the Crh gene and to regulate its expression (McGill et al. 2006), mutations in the Mecp2 gene are likely to produce behavioural effects similar to the ones produced by long-term demethylation of the Crh gene. Reduced sociability (Moretti et al. 2005) and reduced preference for sucrose, which is considered as an index of anhedonia (Deng et al. 2010) have been, in fact, reported in fully symptomatic Mecp2-308 mice, thus suggesting a potential role of Crh gene expression abnormalities as neurobiological correlates of those behavioural alterations in this RTT mouse model. Further studies aimed at evaluating this hypothesis are however needed.

Drug challenges constitute a very useful tool to indirectly investigate the functionality of targeted neurochemical systems (Bignami 1996). In Mecp2-308 mice, we observed an exaggerated behavioural response to a challenge with the prototype monoaminegic stimulant d-amphetamine and a decreased behavioural response to scopolamine, a direct cholinergic muscarinic drug, when compared to wt animals (De Filippis et al. 2010).

When challenged with d-amphetamine, fully symptomatic (5 months old) Mecp2-308 mice showed a marked increase in both locomotion and stereotyped behavioural syndrome (Laviola et al. 1994; Laviola et al. 2004), thus suggesting an exaggerated activation of both the mesolimbic and the mesostriatal dopamine pathways in this RTT mouse model (see(De Filippis et al. 2010)). In an interesting paper (Deng et al. 2010), which followed our report (De Filippis et al. 2010), enhanced locomotor activity after acute amphetamine administration was confirmed in Mecp2-308 mice; however, while repeated daily injection of amphetamine over 5 consecutive days drove a progressive increase in locomotion in wt mice, no significant enhancement was detected in Mecp2-308 mice over the same period, raising the possibility that mechanisms of locomotor sensitization may be impaired in Mecp2-308 mice. The same mice also showed no significant preference for the drug-paired chamber in a condition place preference test and a deficient amphetamine-induced structural plasticity of nucleus accumbens (NA) dendritic spines.

Importantly, acute viral manipulation of Mecp2 expression in the NA in adult mice bidirectionally modulated amphetamine-induced conditioned place preference, and locomotor activity in mice expressing the shRNA against Mecp2 were significantly enhanced after both acute and repeated administration of amphetamine (Deng et

al. 2010). One explanation for the differences between requirements for Mecp2 in the adult NA and the behavioural phenotypes in Mecp2-308 mice is that some of the behavioural effects of the constitutive mutation may arise secondary to changes in mesolimbocortical circuit development or may be the result of compensatory changes deriving from the absence of the Mecp2 gene during development. As a whole, these data reveal new roles for Mecp2 both in mesolimbocortical circuit development and in the regulation of psychostimulant-induced behaviours, and highlight the importance of taking into account, when studying genetically modified mouse models, the implications of constitutive genetic alterations.

As well as an amphetamine challenge allowed us to identify a role for dopamine pathways in RTT pathophysiology (De Filippis et al. 2010), the identification of a reduced responsiveness to scopolamine in Mecp2-308 mice (Alleva et al. 1985; Laviola et al. 2006) provided us a first indirect indication of impairments in the central cholinergic function (Ricceri et al. 2011). This hypothesis was subsequently confirmed by the observation that choline acetyl-trasferase (ChAT) enzymatic activity is decreased in the striatal region in Mecp2-308 animals and by the paradoxical increase in nerve growth factor (NGF) levels in the hippocampus of mutant mice (see (Ricceri et al. 2011)), which suggest a widespread dysfunction of central cholinergic system.

Given the number of important roles played by cholinergic transmission in the CNS, including sensory and motor processing, sleep, nociception, mood, stress response, attention, arousal, memory, motivation and reward (Perry et al. 1999; Lucas-Meunier et al. 2003; Smythies 2005), our study, which is the first report of cholinergic hypofunction in a mouse model of RTT, paves the way to further ones aimed at exploring the involvement of central cholinergic system in RTT symtomatology for a better understanding of the disease.

Among the many modulators of neural plasticity, nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) play a major biological role. NGF and BDNF are well-studied neurotrophins involved in the neurogenesis, differentiation, growth and maintenance of selected peripheral and central populations of neuronal cells during development and at adulthood (Alleva et al. 2009; Cirulli et al. 2009; Cirulli et al. 2009). In addition to the aforementioned functions, these neurotrophins are important mediators of synaptic and morphological plasticity (Levi-Montalcini 1987; Thoenen 1995; Lewin et al. 1996). Recently, some step forwards have been made in the understanding of how neurotrophin-mediated changes in cell shape are transduced: BDNF, acting through TrkB, leads to Rac1 activation, a small GTPases of the Rho family which plays key roles in the formation of neuronal axons and dendrites (see (De Filippis et al. 2012; Miyamoto et al. 2006).

The identification of BDNF as a target gene of Mecp2 (Chen et al. 2003; Chang et al. 2006) and the observation that exogenous administration of this neurotrophin can rescue RTT symptomatology (Kline et al. 2010) have generated a great interest in these molecules in RTT research. Importantly, a decrease in BDNF levels has been reported in Mecp2-null mice (Schaevitz et al. 2010).

The observation that BDNF mRNA and protein levels are not altered in Mecp2-308 mice (see (Ricceri et al. 2011)), however, suggest that the truncated form of Mecp2 which is present in our RTT mouse model could retain some of its functions, thus contributing to the milder neurobehavioural phenotype of those mice. To further support this hypothesis, immunohistochemical analyses, performed on brain tissue of Mecp2-308 mice, showed no genotype differences in neuronal dendritic tree and synapses (see (De Filippis et al. 2012)), one of the major anatomical features in the brain of Mecp2-null mice (Belichenko et al. 2009). As clinical studies support the presence of differences in the clinical manifestation of the syndrome in RTT patients carrying different mutations in the Mecp2 gene (Robertson et al. 2006; Bebbington et al. 2008), our results strongly support the need for further studies aimed at elucidating the genotype-phenotype correlation in RTT. Indeed, thanks to the development of international databases, many step forwards have been made in the study of genotype-phenotype correlations in clinical research. The availability of RTT mouse models carrying different mutations in the Mecp2 gene now offers the unique opportunity to preclinical research to uncover the neurobiological correlates of such clinical observations.

Another main finding of the neurobehavioural characterization of Mecp2-308 mice we performed is the presence of evident signs of atrophy in the astrocytes of hippocampus and corpus callosum (De Filippis et al. 2012). Although recent in vitro reports point to a critical role of astrocytes in the pathophysiology of RTT (Nagai et al. 2005; Cahoy et al. 2008; Ballas et al. 2009; Maezawa et al. 2009), this is the first time that alterations in astrocytic cell populations are reported in a RTT mouse model in vivo. Such observation opens a new window of opportunity to RTT research. As a matter of fact, increasing evidence demonstrate that astrocytes play a relevant role in synaptogenesis and neural plasticity (Barres 2008), and in particular in hippocampal forms of synaptic plasticity (Henneberger et al. 2010). Recovering astrocytic function could therefore represent a new appealing strategy to treat RTT symptomatology and Mecp2-308 mice a good mouse model on which to test new therapeutic approaches targeted at improving astrocytic functionality.

7. Final remarks

In conclusion, results from the series of studies here reported provided a better understanding of the neurobehavioural phenotype of Mecp2-308 mice and several biomarkers to be exploited for the evaluation of new therapeutic approaches. The importance of combining different approaches when studying behavioural disorders (i.e. behavioural analyses and neurobiological evaluations) is also suggested. The understanding of basic processes underlying the behavioural phenotype of animal mod-

els is indeed particularly relevant for the prevention and treatment of psychopathologies and, more in general, for understanding how neural systems dysfunction under pathological conditions.

In addition to the translational value of the studies here reported, the utility of an ethologically oriented approach in the study of genetically modified mouse models is worth of being emphasized. As a matter of fact, incorporating ethologically based tests (e.g. nest building abilities, see (De Filippis et al. 2012)) into an otherwise standard behavioural neuroscience program offers the unique opportunity to gain insight into the effects of gene mutations on behaviours spontaneously emitted by rodents.

In conclusion, the discovery of a monogenic origin for RTT and the subsequent generation of RTT mouse models have actually provided to basic research the necessary tools for understanding, and hopefully treating, this devastating syndrome. Many steps forward have been made so far and, more importantly, many indications are available that this research field could rapidly progress and that the translation of basic research into efficient clinical, pharmacological and non-pharmacological interventions for RTT patients can be achieved. Strong evidences in Mecp2-null models in fact suggest that RTT is reversible even at a mature stage. Moreover, as recent studies support the presence of subtle deficits during that phase previously regarded as asymptomatic, the pre-symptomatic stage is now expected to provide an early window of opportunities on which potential therapeutic approaches could be tested. Many research efforts are now expected in order to guarantee to RTT girls to experience a better quality of life.

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