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MONOAMINE DEFICITS IN THE BRAIN OF METHYL-CpG BINDING PROTEIN 2 NULL MICE SUGGEST THE INVOLVEMENT OF THE CEREBRAL CORTEX IN EARLY STAGES OF RETT SYNDROME

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Abstract—Rett syndrome is a neurodevelopmental disorder caused by mutations in the methyl-CpG binding protein 2 gene (*MECP2*). Several neural systems are affected in Rett, resulting in an autonomic dysfunction, a movement disorder with characteristic loss of locomotor abilities and profound cognitive impairments. A deregulation of monoamines has been detected in the brain and cerebrospinal fluid of both Rett patients and a Rett syndrome murine model, the *Mecp2* knock-out mouse. Our goal was to characterize the onset and progression of motor dysfunction in *Mecp2*$^{tm1.1Bird}$ knock-out mice and the possible neurochemical alterations in different brain regions potentially playing a role in Rett-like pathophysiology, at two different time-points, at weaning (3 weeks old) and in young adults when overt symptoms are observed (8 weeks old). Our results revealed significant age- and region-dependent impairments in these modulatory neurotransmitter systems that correspond well with the motor phenotype observed in these mice. At 3 weeks of age, male *Mecp2* knock-out mice exhibited ataxia and delayed motor initiation. At this stage, noradrenergic and serotonergic transmission was mainly altered in the prefrontal and motor cortices, whereas during disease progression the neurochemical changes were also observed in hippocampus and cerebellum. Our data suggest that the deregulation of norepinephrine and serotonin systems in brain regions that participate in motor control are involved in the pathophysiology of Rett syndrome motor phenotypes. Moreover, we highlight the contribution of cortical regions along with the brainstem to be in the origin of the pathology and the role of hippocampus and cerebellum in the progression of the disease rather than in its establishment.

Key words: RTT, neurodevelopment, autism, mental retardation, norepinephrine, serotonin.

Rett syndrome (RTT) is an X-linked neurological disorder caused by mutations in the methyl-CpG binding protein 2 gene (*MECP2*) (Amir et al., 1999). RTT patients appear to develop normally until 6–18 months of age, and thereafter they develop a progressive syndrome characterized by cognitive and behavioral disturbances, autonomic dysfunction and motor impairment (Hagberg et al., 1983, 2002). Several lines of evidence indicate that dysfunction of monoaminergic systems may contribute to the neuropathology of RTT. Indeed, clinical and polysomnographic studies suggest that the primary lesion of RTT involves the raphe nuclei and the locus coeruleus (Nomura et al., 1987), origin of some monoamine transmitter systems. Studies in RTT patients' brains (post mortem) and cerebrospinal fluid (CSF) (*in vivo*) have revealed alterations (mostly reductions) in the levels of biogenic amines (Zoghbi et al., 1985, 1989; Percy et al., 1987; Perry et al., 1988; Lekman et al., 1989, 1990; Lappalainen and Riikonen, 1996; Wenk and Mobley, 1996; Blue et al., 1999a, b; Ramaekers et al., 2003; Ormazabal et al., 2005; Temudo et al., 2009) (see Suppl. Table S1). However, the data from different reports are conflicting, making it difficult to reach a clear conclusion.

In recent years, different knock-out (ko) and transgenic mouse models of RTT have been created, that recapitulate several aspects of the disorder (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002; Pelka et al., 2006; Ricceri et al., 2008). All these mice exhibit RTT-like features: a period of apparently normal development followed by a severe, progressive neurological dysfunction that includes abnormal gait, irregular breathing, tremors and hypoactivity. Null males die at 8–12 weeks of age while late truncations of MeCP2 allow the mice to survive to adulthood.

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Abbreviations: Adrα2a, adrenergic receptor α2a; Adrβ2, adrenergic receptor β2; ANOVA, analysis of variance; BSA, bovine serum albumin; CSF, cerebrospinal fluid; DA, dopamine; DAB, diaminobenzidine; D/MRN, dorsal/medial raphe nuclei; DOPAC, 3,4-diydroxyphenylacetic acid; EDTA, ethylenediaminetetraacetic acid; HPLC/EC, high performance liquid chromatography/electrochemical detection; Hprt, hypoxanthine guanine phosphoribosyl transferase; Htr1a/2a/2b/3a, Serotonin receptor1a/2a/2b/3a; HVA, homovanillic acid; H2O2, hydrogen peroxide; KO, knock out; MAO A/B, monoamine oxidase A/B; MECP2, methyl-CpG binding protein 2; NE, norepinephrine; NET(Slc6a2), norepinephrine transporter; nNOS, neuronal nitric oxide synthase 1; PBS, phosphate buffered saline; PFA, paraformaldehyde; PMSF, phenylmethylsulfonyl fluoride; PND, postnatal day; RTT, Rett syndrome; SEM, standard error of the mean; SERT(Slc6a4), Serotonin transporter; SN-VTA, substantia nigra-ventral tegmental area; Sytl, synaptotagmin 1; Th, tyrosine hydroxylase; Tph2, tryptophan hydroxylase 2; Vamp2, vesicle-associated membrane protein 2; WT, wild type; 5-HIAA, 5-hydroxyindoleacetic acid.

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By studying the postnatal neurodevelopmental profile of *Mecp2* ko mice (Guy et al., 2001), we have shown that the first, subtle motor problems became evident already during the early postnatal weeks (Santos et al., 2007). Therefore, this mouse model constitutes a good tool to study the motor impairments of RTT and their neurobiological substrates at early stages of the disease. Early postnatal changes were also recently found in the *Mecp2*-308 mouse model of Rett (De Filippis et al., 2010).

A neurochemical study performed in total brain homogenates of *Mecp2* ko males and their wild type (wt) littermates revealed that the concentration of the biogenic amines norepinephrine (NE), dopamine (DA) and serotonin (5-HT) was lower in *Mecp2* ko than in wt animals, these differences being accentuated with increasing age (Ide et al., 2005). Two other studies (Viemari et al., 2005; Roux et al., 2008) linked the breathing disturbances in *Mecp2* ko animals to a deficiency in noradrenergic and serotonergic modulation of the medullary respiratory circuitry. Given the role of monoaminergic systems in motor behavior, we hypothesized that the motor phenotype of RTT might also be due to impairments in monoaminergic neurotransmission in brain regions involved in the control of movement. To address this hypothesis, we used *Mecp2* ko and wt mice at different ages (and therefore representing different stages of the disease), and analyzed different aspects of motor behavior and monoamine levels in several brain regions that potentially play a role in RTT motor pathology.

EXPERIMENTAL PROCEDURES

Animals

Mecp2$^{tm1.1Bird}$ mice were created in the laboratory of A. Bird (Guy et al., 2001) and made available to the scientific community through the Jackson Laboratory (ME, USA). Briefly, *Mecp2*$^{tm1.1Bird}$ mice were generated, using the Cre-loxP technology, by Cre-mediated deletion of exons 3 and 4 of the *Mecp2* gene and maintained on a C57BL/6J background. The deletion was confirmed in the CNS by Northern blot (RNA), and by Western blot the protein was totally absent in the hemizygous males. In the heterozygous females the protein is reduced by half and is normally distributed in the CNS (Guy et al., 2001). The colony was maintained by initially crossing heterozygous *Mecp2*$^{tm1.1Bird}$ females with C57BL/6J males, also purchased from the Jackson Laboratory. Thereafter, heterozygous females were mated with wt males from the colony. The littermates served as wt controls for all experiments. Around postnatal day (PND) 21–23, pups were weaned and group housed (3–5 animals) by sex in standard laboratory cages, filled with sawdust and cardboard rolls. Animals were maintained in an animal facility with controlled temperature at 22 °C, on a 12-h light/12-h dark cycle and with standard food pellets (Mucedola, MI, Italy), and water *ad libitum*. DNA was extracted from tail tips using the Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). Genotype was determined by polymerase chain reaction according to a protocol provided by the Jackson Laboratory for this strain. All experiments were performed in accordance with the European Communities Council Directive, 86/609/EEC. We took into consideration minimizing the number of animals used in the study and their suffering.

Behavioral testing

Behavioral tests were performed using 3-week-old male animals from eight different litters immediately after weaning (ko, $n=10$ and wt, $n=15$), and in the same group at 8 weeks of age (ko, $n=5$ and wt, $n=13$). The differences in the number of mice used at 3 and 8 weeks of age are due to the death of some mice at about 8 weeks of age. All behavioural tests were performed during the same circadian period (between 9 AM and 6 PM).

Open field. To evaluate their motor activity animals were placed in the center of an arena (43.2×43.2 cm^2; MedAssociates Inc., St. Albans, VT, USA) with transparent walls and brightly illuminated and their behavior was observed for 5 min. The following parameters were collected using MedAssociates software: total distance travelled, resting time, and distance travelled and time spent in the center of the arena versus the peripheral area. An observer scored the number of rears and the time spent exploring vertically. The time taken (1) to start walking and (2) to reach the wall of the arena was also registered, as measures indicative of movement initiation. The apparatus was cleaned between subjects with a solution of 10% ethanol.

Gait onset. This test was used to assess movement initiation. Animals were placed in the center of a circle (diameter 13 cm) drawn on a white paper sheet and the time, in seconds, taken to move all four paws out of the circle was recorded (30 s was the maximum duration of the test).

Footprint pattern. The footprint test was used to compare the gait of *Mecp2* ko with that of wt control mice (Carter et al., 1999). Fore- and hindpaws of mice were painted with purple and black non-toxic paints, respectively, and the animals were guided to walk through a tunnel covered with a white paper sheet. The pattern of three consecutive steps (the first four steps were excluded from the analysis) was analyzed and the following parameters assessed, averaged over consecutive steps (refer to the caption of Fig. 2G (g1–g4) for a detailed explanation of how these measures are derived): (1) forepaw and (2) hindpaw base width, measured as the average distance between left and right front footprints and left and right hind footprints, respectively; (3) stride length (the average distance between each stride); and (4) uniformity of step alternation (distance from left or right front footprint/hind footprint overlap). These values were determined by measuring the perpendicular distance of a given step to a line connecting its opposite preceding and proceeding steps.

Neurochemical determinations

Levels of monoamines and their metabolites were measured by high performance liquid chromatography, combined with electrochemical detection (HPLC/EC).

Naive male *Mecp2* ko and their wt controls were killed by decapitation at three (wt, $n=10$; *Mecp2* ko, $n=9$) or eight (wt, $n=7$; *Mecp2* ko, $n=9$) weeks of age and their brains were rapidly removed, snap frozen in isopentane cooled in liquid nitrogen and stored at −80 °C until neurochemical determinations.

For HPLC/EC analysis, prefrontal cortex, motor cortex, caudate-putamen, hippocampus, ventral mesencephalon (comprising substantia nigra and ventral tegmental area, SN-VTA), dorsal and medial raphe nuclei (D/MRN), vestibular area, and cerebellum were dissected. Dissection was performed on ice with the help of a 2× magnifying lens, following a stereotaxic brain atlas (Franklin and Paxinos, 1997). Once dissected, the tissue was kept in 150 μl 0.2 N perchloric acid and stored at −80 °C. The day before the neurochemical determination, samples were moved to −20 °C and on the day of the analysis samples were defrosted on ice, sonicated for 2 min and centrifuged at 5000 rpm for 3 min at 4 °C. The supernatant was collected and filtered through a 0.22 μm nylon filter (micro centrifuge SpinX HPLC filter from Costar, Corning, NY, USA) at 10,000 rpm for 5 min at 4 °C. Aliquots of 50 μl were injected into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (monobasic) (pH=3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/l) and Na-EDTA (40