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A codon-optimized *Mecp2* transgene corrects breathing deficits and improves survival in a mouse model of Rett syndrome

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ABSTRACT

Rett syndrome (RTT) is a severe X-linked neurodevelopmental disorder that is primarily caused by mutations in the methyl CpG binding protein 2 gene (*MECP2*). RTT is the second most prevalent cause of intellectual disability in girls and there is currently no cure for the disease. The finding that the deficits caused by the loss of *Mecp2* are reversible in the mouse has bolstered interest in gene therapy as a cure for RTT. In order to assess the feasibility of gene therapy in a RTT mouse model, and in keeping with translational goals, we investigated the efficacy of a self-complementary AAV9 vector expressing a codon-optimized version of *Mecp2* (AAV9-MCO) delivered via a systemic approach in early symptomatic *Mecp2*-deficient (KO) mice. Our results show that AAV9-MCO administered at a dose of 2×10^{11} viral genome (vg)/mouse was able to significantly increase survival and weight gain, and delay the occurrence of behavioral deficits. Apneas, which are one of the core RTT breathing deficits, were significantly decreased to WT levels in *Mecp2* KO mice after AAV9-MCO administration. Semi-quantitative analysis showed that AAV9-MCO administration in *Mecp2* KO mice resulted in 10 to 20% Mecp2 immunopositive cells compared to WT animals, with the highest Mecp2 expression found in midbrain regions known to regulate cardio-respiratory functions. In addition, we also found a cell autonomous increase in tyrosine hydroxylase levels in the A1C1 and A2C2 catecholaminergic Mecp2 + neurons in treated *Mecp2* KO mice, which may partly explain the beneficial effect of AAV9-MCO administration on apneas occurrence.

1. Introduction

Rett syndrome (RTT) is a severe X-linked neurodevelopmental disorder that is primarily caused by mutations in the methyl CpG binding protein 2 gene (*MECP2*) (Amir et al., 1999) and is the second most prevalent genetic cause of intellectual disability in girls (Hagberg, 1995; Naidu, 1997; Neul et al., 2010). It is a disease affecting not only the CNS (profound cognitive and motor deficits) but also the motor and autonomic functions (including severe breathing abnormalities). Currently, treatments are aimed at alleviating symptoms and there is no cure for RTT (Katz et al., 2012; Lombardi et al., 2015).

Mecp2 was first characterized as a transcriptional repressor (Meehan et al., 1992) but has since been involved in additional pathways such as transcriptional activation, mRNA splicing and miRNA processing (Samaco and Neul, 2011). In addition, *Mecp2* was also reported to be a genome-wide regulator of chromatin structure and a transcriptional regulator [see (Lyst and Bird, 2015) for review], which highlights the complexity of its molecular and cellular functions and explains why therapeutic approaches other than replacing *MECP2* may only partially improve RTT symptoms.

Because most *MECP2* mutations are sporadic (Amir et al., 1999), RTT can only be diagnosed after the first clinical signs appear. Therefore, any therapeutic intervention, such as gene therapy, would occur after disease onset. It was previously shown that reactivation of *Mecp2* rescued adult diseased RTT mice (Guy et al., 2007; Robinson et al., 2012) which indicates that, at least in animal models, the deficits caused by a loss of *Mecp2* are reversible. These finding suggest that gene therapy after the disease has started might still be beneficial for RTT patients.

Adeno-associated viruses (AAVs) are the vectors of choice for gene therapy because they are nonpathogenic, present a low immunogenicity and can express their transgene for a long period of time [see (Asokan et al., 2012) for review]. Among the different clades of viruses, AAV9 is of great interest for RTT gene therapy as it was shown to cross the blood brain barrier and infect brain cells after intravenous injection in both rodents and primates (Duque et al., 2009; Foust et al., 2009; Gray et al., 2011b).

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In the present study, we therefore investigated the therapeutic effect of gene therapy in a RTT mouse model, the *Mecp2* deficient (*Mecp2* KO) male mouse (Guy et al., 2001).

2. Results

2.1. The short Mecp2 promoter preferentially directs GFP expression in neurons after scAAV9 intravascular administration

In order to improve transgene expression in target cells (Mecp2 expressing cells), we used a self-complementary (sc) AAV9 vector that was previously shown to have an increased transduction efficiency over its single stranded counterpart (McCarty et al., 2001). In addition, to comply with the 2.2 kb maximum packaging capacity of the scAAV vector, we used the short mouse *Mecp2* endogenous promoter (pME) that was previously shown to preferentially drive the expression of a transgene in neuronal tissues (Adachi et al., 2005; Gray et al., 2011a).

Thirty day-old wild-type (WT) male mice were intravenously injected with a scAAV9-pME-GFP or scAAV9-CMV-GFP vector (control vector) at a dose of 5×10^9 viral genome (vg)/g BW. Three weeks after injection, the number of GFP expressing (GFP+) cells in the injected mouse brains was assessed by immunostaining (Fig. 1). A semi-quantitative analysis in 4 different brain areas showed a significantly lower number of GFP+ cells in the scAAV9-pME-GFP group compared to scAAV9-CMV-GFP (Fig.1C). This difference was mostly due to the CMV promoter directing GFP expression in non-neuronal cells as the number of GFP+ neuron like cells after injection of either vectors was similar in all brain areas, except the striatum (Fig. 1D). The neuronal specificity of the scAAV9-pME-GFP vector was confirmed by double fluorescent immunostaining using a neuron-specific marker (NeuN, Fig. 1N–P and supplemental Fig. 1). As previously reported by others (Dufour et al., 2014; Foust et al., 2009), the systemic administration of the scAAV9-CMV-GFP vector resulted in various cell co-expressing GFP and cell-specific markers such as NeuN (neuronal marker, Fig. 1H–J and supplemental Fig. 1), s100b (glial marker, supplemental Fig. 2), GFAP (astrocytic marker, supplemental Fig. 3) or CD31 (endothelial cell marker, supplemental Fig. 4). We did not observe any GFP expression in IBA1+ microglial cells in either vector-injected groups (supplemental Fig. 3).

2.2. Intravascular injection of a self-complementary AAV9 vector expressing a codon-optimized version of Mecp2 delays behavioral deficits worsening and increases survival in Mecp2 KO mice

In order to optimize the expression of Mecp2, we elected to use a codon-optimized version of the major *Mecp2* brain isoform [*Mecp2e1* (Dragich et al., 2007)] termed MCO and whose design involved the use of the most frequently used codons, the adjustment of GC content in order to prolong mRNA half-life as well as the replacement of negative cis-acting sites (Raab et al., 2010). Based on the GFP expression data obtained after injection of the control vectors (Fig. 1), we chose to increase the experimental vector dose to 2×10^{11} vg/mouse (1.6×10^{10} vg/g BW). Therefore, 30 day-old *Mecp2* KO mice were intravenously injected with a scAAV9 vector expressing MCO under the regulation of the short *Mecp2* promoter, termed AAV9-MCO. These mice (AAV9-MCO KO group) and their littermate controls (WT and/or *Mecp2* KO mice) were examined at different ages to monitor the progression of behavioral deficits (Fig. 2).

In early symptomatic, 35 day-old male mice (P35), there was a trend in decreased sensorimotor function in *Mecp2* KO mice compared to WT (accelerated rotarod test, Fig. 2A. $P = 0.06$, one-way ANOVA). We did not observe any significant difference in exploratory behavior (open field test, Fig. 2D–G) in the *Mecp2* KO group compared to the AAV9-MCO KO or WT groups. In the elevated plus maze test, P45 *Mecp2* KO mice were hypoactive (Fig. 2C) and showed decreased anxiety levels (Fig. 2B) as previously reported in another *Mecp2*-deficient mouse model (Pelka et al., 2006). This behavioral deficit was not improved by AAV9-MCO administration in *Mecp2* KO mice (Fig. 2B, C). In late symptomatic *Mecp2* KO mice (P55), we found significant alterations in sensorimotor function (Fig. 2A) as well as in exploratory behavior (Fig. 2D-G) when compared to WT mice. In AAV9-MCO treated *Mecp2* KO mice, the occurrence of these behavioral deficits seemed to be delayed as their performance on the rotarod (Fig. 2A) and in the open field (Fig. 2D-F) were not significantly different form the WT mice, except for the total vertical activity parameter that was still significantly decreased (Fig. 2G).

All mice were weighted three times a week and assessed for survival. *Mecp2* KO mice maintained on a C57Bl/6 background are lighter than their WT littermates (Guy et al., 2001) and a sudden drop in their weight curve usually marks the worsening of RTT symptoms. At the beginning of the study (P30), *Mecp2* KO mice already showed a significantly lower body weight than WT controls and there was no significant difference in weight between the treated and untreated *Mecp2* KO groups (Fig. 3A). While the *Mecp2* KO mice stopped gaining weight by P45, the AAV9-MCO KO mice kept gaining weight and were significantly heavier than the *Mecp2* KO mice at P50 and P60 (Fig. 3A). In order to better tease out the effect of AAV9-MCO on weight gain, we examined how long treated and untreated *Mecp2* KO mice kept gaining weight (age at peak weight) and recorded their maximum weight (Wmax). We found that AAV9-MCO KO mice kept gaining weight significantly longer than the untreated *Mecp2* KO ones (age at peak weight, 83d ± 9.1d vs 54.1d ± 4.3d, $P < 0.021$, AAV9-MCO KO vs KO by Mann-Whitney U statistics test). In addition, treated *Mecp2* KO mice reached a Wmax that was significantly higher than that of untreated *Mecp2* KO (Fig. 3A).

We also observed a significant increase in the survival median period in the AAV9-MCO KO group compared to the untreated one (99d vs 56d, respectively, $P = 0.014$, Kaplan-Meier survival analysis, Fig. 3B).

2.3. AAV9-MCO administration to Mecp2 KO mice results in low but widespread Mecp2 expression following a rostro-caudal gradient

After AAV9-MCO injection, Mecp2 expressing (Mecp2+) cells were identified by immunostaining and subsequently quantified by automated counting using the ImageJ *particle analyzing tool* (Fig. 4). As expected, we did not find any Mecp2 expression in the brains of *Mecp2* KO mice (4D, G and 4 J, M). In forebrain regions of AAV9-MCO KO mice, the number of Mecp2+ cells was around 10% of the WT number while it doubled in hindbrain regions (Fig. 4C). As seen with the scAAV9-pME-GFP construct (Fig. 1N–P), almost all Mecp2+ cells in the AAV9-MCO KO mice were neurons co-expressing NeuN (Fig. 4H, N, supplemental Fig. 5). While we could not find any Mecp2+ GFAP+ astrocyte (supplemental Fig. 6A–L), we did find Mecp2+ cells at the gliovascular interface (supplemental Fig. 6a–l).

2.4. AAV9-MCO administration to Mecp2 KO mice prevents the occurrence of apneas and increases TH levels in a cell autonomous manner

Breathing dysfunctions are common in RTT patients, as well as in RTT mouse model (Katz et al., 2009; Viemari et al., 2005), and may be responsible for a quarter of the unexplained sudden death observed in RTT patients (Julu et al., 1997; Kerr et al., 1997). In order to assess the effect of AAV9-MCO on breathing, whole body plethysmography was used to determine breathing parameters in symptomatic *Mecp2* KO mice (P45). As previously reported (Roux et al., 2007; Viemari et al., 2005), *Mecp2* KO mice had a significantly higher number of apneas when compared to WT mice and this abnormality was normalized by AAV9-MCO injection as soon as 15 days post-injection (P45) and seemed to last at least until P80 (Fig. 5). Among the other breathing parameters recorded at P45 (Table 1), the number of hypoventilation, the mean frequency and the variability were found to be significantly different in *Mecp2* KO compared to WT mice. In the AAV9-MCO KO group,