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Recombinant Adeno Associated Viral (AAV) vector type 9 delivery of Ex1-Q138-mutant huntingtin in the rat striatum as a short-time model for in vivo studies in drug discovery

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ABSTRACT

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by dyskinesia, cognitive impairment and emotional disturbances, presenting progressive neurodegeneration in the striatum and intracellular mutant Huntingtin (mHTT) aggregates in various areas of the brain. Recombinant Adeno Associated Viral (rAAV) vectors have been successfully used to transfer foreign genes to the brain of adult animals. In the present study we report a novel in vivo rat HD model obtained by stereotaxic injection of rAAV serotype2/9 containing Exon1-Q138 mHTT (Q138) and Exon1-Q17 wild type HTT (Q17; control), respectively in the right and in the left striatum, and expressed as C-terminal GFP fusions to facilitate detection of infected cells and aggregate production. Immunohistochemical analysis of brain slices from animals sacrificed twenty-one days after viral infection showed that Q138 injection resulted in robust formation of GFP-positive aggregates in the striatum, increased GFAP and microglial activation and neurodegeneration, with little evidence of any of these events in contralateral tissue infected with wild type (Q17) expressing construct. Differences in the relative metabolite concentrations (N-Acetyl Aspartate/Creatine and Myo-Inositol/Creatine) were observed by H1 MR Spectroscopy. By quantitative RT-PCR we also demonstrated that mHTT induced changes in the expression of genes previously shown to be altered in other rodent HD models. Importantly, administration of reference compounds previously shown to ameliorate the aggregation and neurodegeneration phenotypes in preclinical HD models was demonstrated to revert the mutant HTT-dependent effects in our model. In conclusion, the AAV2/9-Q138/Q17 exon 1 HTT stereotaxic injection represents a useful first-line in vivo preclinical model for studying the biology of mutant HTT exon 1 in the striatum and to provide early evidence of efficacy of therapeutic approaches.

1. Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by dyskinesia, cognitive impairment, emotional disturbances and metabolic disorders, predominantly associated with progressive neurodegeneration of medium-spiny neurons in the striatum and with the presence of intracellular Huntingtin (HTT) aggregates in various areas of the brain.

The mutation responsible for HD has been identified as a CAG expansion within the Exon 1 of HTT gene (IT15, located on chromosome 4p16.3), which is translated into a polyQ stretch at the protein level (The Huntington's Disease Collaborative Research Group's, 1993; reviewed by Ross and Tabrizi, 2011; Ha and Fung, 2012; Bates et al., 2015). Individuals with 35 CAG repeats or fewer do not develop HD, while repeats of 40 and above are invariably associated with disease appearance (Myers et al., 1988).

Currently there is no effective treatment for preventing or delaying the disease, which typically sets in at around 35–45 years of age and progresses toward death within 10–20 years after the appearance of

Abbreviations: HD, Huntington's disease; mHTT, mutant Huntingtin; rAAV, recombinant Adeno Associated Virus; LV, lentivirus; tNAA, total N-Acetyl Aspartate; Myo-Ins, myoinositol; GFP, green fluorescent protein; NeuN, neuronal nuclei; DARPP-32, dopamine- and cyclic AMP-regulated phosphoprotein; ChAT, choline acetyl transferase; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule 1; PBST, phosphate buffered saline-Triton-X100; BSA, bovine serum albumin; DAB, diaminobenzidine; ROI, region of interest.

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the first clinical symptoms. It is crucial from a drug discovery prospective to create animal models that, in addition to presenting symptoms typical of striatal neurodegeneration, also recapitulate the genetic and molecular mechanisms underlying the degenerative processes of the human pathology. To that end, the combination of knowledge of the genetic basis of the disease and the emergence of transgenic and gene transfer technologies has allowed the creation of animal models of HD (from the invertebrate C. Elegans to primates) that recapitulate the genetic defect found in humans and are able to reproduce phenotypes reminiscent of HD such as nuclear huntingtin inclusions, neurodegeneration, motor deficits and cognitive impairment (Menalled, 2005, Ramaswamy et al., 2007; Fecke et al., 2009). In most cases, these models are based on mutant huntingtin bearing ca. 100 or more glutamines (e.g. R6/2, BACHD, YAC128 and Knock-In mice, with ca. 130, 97, 128 and 111 polyglutamine repeats respectively; Ferrante, 2009; Menalled and Brunner, 2014). Polyglutamine expansions larger than observed in adult-onset HD and closer to those observed in the earlier onset, more aggressive juvenile HD are often required to create robust aggregation and pathology in rodent models. However, despite the availability of several models and intensive efforts on the part of drug developers to date no clinical data exists to support the translational value of preclinical models for HD patients. Mammalian models of HD are dominated by the use of rodents (especially mice) which historically represent the mammalian species of choice for the generation of genetic models. Several rodent lines have been generated expressing mutant huntingtin as transgenic (either as full length protein or as N-terminal fragments) or knock-in models, which in some cases have been extensively characterized at the molecular, histopathological as well as behavioral/cognitive level. The severity of the phenotypes produced as well as their resemblance to human HD is typically stronger in models expressing N-terminal fragments, and in particular in those expressing the protein fragment encoded by exon 1 (Mangiarini et al., 1996; Gray et al., 2008;, Slow et al., 2003; etc.; reviewed in Lee et al., 2013). In spite of the usefulness of these models to investigate disease mechanisms, drug efficacy trials in most of these rodent models (including exon 1 models) require labor-, time- and cost-intensive studies, lasting several months to more than a year and typically requiring relatively large cohorts of animals. The availability of relatively more rapid, less resource intensive in vivo models, reproducing key aspects of the human pathology and bridging the gap between in vitro neuronal models and the more pathophysiologically relevant but low-throughput genetic models would greatly facilitate the evaluation of therapeutic approaches at an early stage of the drug discovery process. In vivo viral gene transfer models represent an opportunity to address this need.

Recombinant Adeno Associated Viral (rAAV) vectors have been successfully used to transfer genes in a variety of tissues, including brain, in adult animals (Burger et al., 2004; Tenenbaum et al., 2004, and McFarland et al., 2009; Huda et al., 2014). Moreover rAAV vectors have also been widely used in clinical trials for neurodegenerative diseases and have been shown to be more effective at transducing certain brain regions compared to lentiviral vectors (de Backer et al., 2010). The first rAAV-based HD model employed to express along CAG repeat fused to green fluorescent protein (GFP) in the rat striatum, described a rapid formation of fibrillar, cytoplasmic and ubiquinated nuclear polyglutamine aggregates as well as neurotoxicity (Senut et al., 2000).

In the present study we used rAAV2/9, expressing Exon 1 HTT carrying 17 or 138 CAG repeats (wild type and mHTT, respectively) to implement and optimize a rat model of HD. As the envisaged model was of an acute nature, a long (Q138) polyglutamine repeat was selected in order to maximize chances of eliciting a robust pathology-relevant response and for polyQ length coherence with one of the transgenic mouse HD models most widely employed for drug screening, namely the R6/2 mouse (Mangiarini et al., 1996). AAV2/9-Exon1-GFP/Q138 (AAV9-Q138) was injected in the right striatum and AAV2/9-Exon1-GFP/Q17 (AAV9-Q17) in the left striatum as control and neurodegeneration and neuroinflammation markers were evaluated by immunohistochemical analysis. In order to test whether our model could recapitulate the hallmarks of the transcriptional imbalance found in HD patients and other models, we also performed qRT-PCR on AAV injected striata on selected genes known to be dysregulated in HD. Magnetic Resonance Imaging and ^{1}H MR Spectroscopy was carried out to evaluate the morphological and metabolic changes induced by mHTT expression. Finally, we validated this in vivo model as a tool for drug screening by demonstrating reduction of the phenotypic responses to mHTT by pharmacological treatment with a tool compound known to be active in reducing aggregation and neurodegeneration in preclinical HD models, namely the transglutaminase inhibitor Cystamine (Dedeoglu et al., 2002; Van Raamsdonk et al., 2005).

2. Methods

2.1. Plasmids construction and rAAV particles preparation.

The exon1 of human HTT gene (GenBank: L27350.1) was amplified by proofreading PCR using specific primers carrying an EcoRI site at the 5′-end and a BamHI site at the 3′-end, using as template a full-length HTT cDNA including a mixed CAG/CCA repeats, coding for a poly Q tract (Wyttenbach et al., 2001; Smith et al., 2014).

The amplicons, named exon1-HTT-Q17 and exon1-HTT-Q138, respectively, were cloned in frame with AcGFP into the pAcGFP-N1 vector (Clontech, NJ USA) fused at C-terminal end of exon 1. The obtained AcGFP-exon1-HTT-Q17/Q138 fragments were then inserted into pcDNA3.1zeo + vector (Invitrogen, UK). The final expression vectors were obtained subcloning the cDNA sequence for AcGFP-exon1-HTT-Q17/Q138 from pcDNA3.1zeo + into pAAV-CAG-cis plasmid (Vector Biolabs, PA). The oligonucleotides used for PCR amplification are listed as follow:

Primer EcoRI-HTT-For: 5′-AAGAATTCACCATGGCGACCCTGGAA-3′.

Primer BamHI-HTT-exon1-Q17/Q138 –Rev: 5′-AAGGATCCCCTCGG TGCAGCGGCTCC-3′.

AAV particles were prepared by Vector Biolabs (Philadelphia, PA USA).

2.2. Animals

Forty four female Wistar rats (175-200 g) (Harlan Italy) were used in this study. The animals were housed in a controlled temperature (20–24 °C) and humidity (40–70%) room maintained on a 12 h dark/light cycle. Animals were placed in individually ventilated solid floor plastic cages (IVC Sealsafe® Plus GR900, Tecniplast, Italy), 3 animals/cage. Food and water were available *ad libitum*. Animal experiments were carried out in conformity to the guidelines of the European Community's Council for Animal Experiments (86/609/EEC and 2010/63/EU) and the Principle of Laboratory Animal Care. All efforts were made to minimize the number of animals used and their suffering. In the present study 10 animals were used in a pilot study to evaluate the development of infection and formation of aggregates; they were sacrificed 48 h, 7 d, 14 d and 21 d after surgery (N = 2/3 per groups). The remaining 34 rats were sacrificed at 21 d after surgery according to the scheme reported in supplementary information (Fig. 1S) and used for morphological determination and reference compound treatment (N = 26), transcriptional analysis (N = 3) and Magnetic Resonance Imaging and H1 MR Spectroscopy determinations (N = 5).

2.3. Drugs

2.3.1. Cystamine dihydrochloride

Cystamine dihydrochloride (Sigma Chemical Co., MO, USA 333 mg/mL) was dissolved in Dulbecco's PBS and used to fill osmotic minipumps (2ML4, Alzet, CA, USA) for the subcutaneous deliver of 100 mg/Kg/day of the compound continuously for 21 days.