Gene therapy-based modeling of neurodegenerative disorders: Huntington's disease

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Summary

Huntington's disease is a fatal neurodegenerative disease characterised by impairments in motor control, and cognitive and psychiatric disturbances. In this chapter, viral vector-mediated approaches used in modelling the key neuropathological features of the disease including the production of abnormal intracellular protein aggregates, neuronal dysfunction and degeneration and motor impairments in rodents are described.

Key words: viral vector, huntingtin, transgenic, genetic, neurons, neurodegenerative disease

Running head: Viral vector-based models of Huntington's disease

1. Introduction

Huntington's disease (HD) is a progressive and devastating neurodegenerative disorder characterised by a triad of symptoms including motor dysfunction, cognitive decline and psychiatric disturbances. There is currently no effective treatment for HD, a disease that progresses towards death within approximately 20 years of disease onset [1]. HD is inherited in an autosomal dominant manner, with the underlying genetic mutation being an expansion of a CAG repeat sequence in exon 1 of huntingtin (*HTT*). This results in the production of huntingtin protein (HTT) with an expanded polyglutamine (polyQ) tract. Expansion to greater than 36 CAG repeats results in the disease, with increased tract lengths associated with an earlier disease onset. Current hypotheses posit that HD could be caused by the dominant-negative action of mutant HTT resulting in a loss of function of the wild-type protein, but increasing evidence suggests a toxic gain of function of mutant HTT (mHTT) is more likely to be causative of HD pathogenesis. The key neuropathological hallmarks of HD are abnormal intracellular aggregates composed of mHTT and other sequestered proteins including transcription factors, neuronal dysfunction and neurodegeneration [2]. The brain regions most profoundly affected are the striatum, where extensive loss of GABAergic projection neurons results in significant atrophy of the striatum. Neuronal loss resulting in the thinning of the cortical layers is also found, [3] with the hippocampus, hypothalamus and substantia nigra also affected [4].

Various animal models of HD have been developed that reproduce specific neuropathological features of the disease. There is no single model that fulfils the criteria of modelling all aspects of the human disease, with each type of model having

specific strengths but also limitations [5]. Early studies utilised specific neurotoxins that induce excitotoxicity (glutamate-, kainic acid-, quinolinic acid) [6-9]) or impair mitochondrial function (3-nitroprionic acid, malonate [10-12]) to produce the characteristic degeneration of neurons in the striatum in rodents and primates. These models have largely been superseded by genetic approaches that aim to emulate the molecular pathogenic mechanisms underlying HD. Transgenic animal models including C. elegans [13,14], Drosophila [15-17] and mice that express full-length, N-terminal fragments of mHTT or knock-in of mHTT sequences into the endogenous *Htt* locus of the animal have been developed [18-22]. Specific hallmarks of the human disease are reproduced including nuclear and neuritic aggregates that are ubiquitinated, neuronal dysfunction and specific impairments in motor function. Of note, although striatal atrophy and neuronal cell death has been reported in several mouse models [23-25], no striatal neuronal cell death is found in other transgenic rodent models [26,27].

Viral vector-based modeling of HD offers several advantages over traditional transgenic approaches. Expression cassettes varying in truncated N-terminal fragments and CAG repeat length under the control of regulatable elements or cell or tissue-specific promoters that direct higher levels of transgene expression than can be achieed in transgenic mouse models can be rapidly developed. Expression cassette design coupled with the engineering of viral capsids can be exploited to to direct transgene expression to neurons or astrocytes. Viral vectors can also be injected at any developmental age in a broad range of species including mice, rats and non-human primates, making it a very versatile tool for disease modelling purposes. Lentiviral, adenoviral and adeno-associated viral (AAV) vectors have been exploited

as gene transfer agents for HD modelling studies both in an *in vitro* and *in vivo* setting as discussed below.

1.1 Using viral vectors to model HD *in vitro*

One advantage of many viral vectors is their ability to efficiently transduce primary neuronal cultures, facilitating studies on the molecular mechanisms underlying mHTT neuropathology. Primary striatal neurons infected with lentiviral vectors expressing N-terminal mHTT expressing 82 polyQ (mHTT82Q) under control of the mouse phosphoglycerate kinase 1 (PGK) promoter develop nuclear and neuritic aggregates that subsequently lead to neuronal dysfunction and toxicity by 6-8 weeks [28]. Aggregate neuropathology is also observed following transduction of primary cortical neurons but in contrast, no signs of neuronal dysfunction were observed, consistent with the selective vulnerability of GABAergic medium spiny neurons to mHTT. The rate of formation of HTT inclusions is considerably slower in cells and is also found in neuritic and cytoplasmic compartments expressing full-length mHTT; in comparison truncated mHTT is found predominantly in the form of nuclear aggregates [29]. Furthermore, Senut and colleagues [30] showed that AAV-mediated expression of GFP fused to expanded CAG repeat lengths in HEK293 cells led to the rapid appearance of GFP-positive cytoplasmic and nuclear aggregates 16 h after infection suggesting that expanded polyQ tracts alone can mediate pathogenic effects.

1.2 Viral vector-based rodent models of HD

Viral vector-based rodent models of HD are a powerful complement to the existing chemical and transgenic mouse and rat models. The most common strategy employed is to inject viral vectors expressing truncated *HTT* transcripts with expanded CAG repeats unilaterally into one striatum, leaving the contralateral side to serve as internal

control for subsequent biochemical and molecular analyses. Neuropathological analyses can be compared against control animals that express a matching *HTT* transcript but with normal repeat length (i.e <36 CAG). Potential new treatments can be initiated either prior or following vector infusion. Most studies have relied on immunohistochemical methods to examine specific aspects of interest in the model including time-dependent changes in mHTT expression levels and intracellular aggregate formation using anti-HTT or antibodies that specifically detect aggregated HTT (e.g EM48), protein partners that interact with HTT and the effects of mHTT on specific striatal neuronal populations (e.g using antibodies to NeuN, calbindin-D28K, DARPP32, choline acetyltransferase (ChAT), parvalbumin, Neuropeptide Y (NPY). Other features such as reactive gliosis can be examined as well as behavioural testing of treated animals to assess any deficits in motor function.

In general, the neuropathological features reported between the different studies is remarkably consistent, with a rapid onset of pathology following vector infusion that progresses to signficant neuronal cell loss in the striatum by 5-12 weeks.. The earliest abnormalities are the appearance of intranuclear neuronal aggregates and inclusions that sequester ubiquitin and other proteins, progressing to neuronal cell loss and striatal atrophy and motor impairment, with variations in the time course of neuropathology dependent on mHTT expression levels, HTT fragment length and CAG repeat length. I will now cover some specific variables that can influence the model.

1.2.1 Viral vectors and vector infusion

Lentiviral[31] and AAV vectors including AAV2[30], chimeric AAV1/2[32] and mosaic AAV1/8[33] vectors have been used to deliver mHTT or polyQ transcripts

into the mouse and rat striatum. AAV1/2 and AAV1/8 vectors transduce a large volume of the striatum compared to AAV2, and possibly lentiviral vectors [31], leading to a greater volume of distribution of transgene expression and production of larger striatal lesions. For example, we found that 35% of the injected striatum was devoid of staining for the neuronal marker calbindin-D28K, DARPP-32 and NeuN by 5 weeks [32]. However, differences in transduction and lesion volumes could also be largely due to differences in the promoters used in the AAV1/2 and AAV1/8 studies compared to the lentiviral vector study; the PGK and CMV promoters were used in a lentiviral context, whereas the strong CMV/chicken beta actin hybrid promoter and neuron- specific enolase (NSE) promoters were used in the AAV studies [32,33]. Indeed we found that the AAV1/2-mediated mHTT expression levels rose to >100-fold that of endogenous rat Hdh expression at 2 weeks, while Drouet et al.,[34] found that mHTT expression levels under control of the PGK promoter in lentiviral vectors was 25-fold higher than endogenous HTT. In a subsequent study, Regulier and colleagues used a tetracycline-regulated lentiviral vectors to express the first 853 amino acids of mHTT. Expression levels were 4-5 fold higher than that achieved using the PGK promoter and induced an early pathological onset and exacerbated the HD neuropathology.[35] One advantage of the tetracycline-regulated system is that it allows conditional expression of mHTT by systemic administration of doxycyline, enabling exploration of the relationship between mHTT protein expression and disease progression.

A snapshot of mHTT dosage effects on the kinetics of aggregate formation or toxicity can often be captured by analyses of brain sections from a 2-5 week time-point, although the specific timing of these appearance of this pathology will vary depending on vector system and mHTT fragment expressed. Typically the highest

levels of transgene are detected 1-2mm from the viral vector injection site, and so the earliest appearance of intranuclear inclusions and neuronal depletion occurs in this region. Surrounding the core region of neuronal depletion, neurons express lower levels of transgene expression presumably reflective of the extent of diffusion of vector from the infusion site. These neurons may show evidence of intracellular aggregates.

In humans with HD, there is preferential loss of GABAergic medium spiny neurons. The demonstration that lentiviral-mediated expression of mHTT leads to selective depletion of DARPP32-positive medium spiny neurons but interneurons in the mHTT-injected rats are largely spared is consistent with that observed in human HD[31]. In contrast, we found that the chimeric AAV1/2 vector used in our study transduced striatal medium spiny neurons but there was increased transduction of cholinergic interneurons compared to other neuronal subtypes. This resulted in toxicity and neuronal death of this interneuron population irrespective of the transgene expressed. In contrast, we found that parvalbumin and NPY-positive interneurons were only susceptible to mHTT [32].

Retrograde and anterograde transport of AAV vectors from the striatal injection site can lead to transgene expression and the appearance of intracellular aggregates in distal basal ganglia regions including the substantia nigra (SN) and globus pallidus [30,32], brain regions where neurodegeneration and atropy are also observed in humans with HD [36]. We found axonal transport of a chimeric AAV1/2 vector expressing exon 1 of HTT with 70Q was associated neuronal cell loss and atrophy of the globus pallidus consistent with that observed in human HD. However, we also observed loss of dopaminergic neurons in the SN pars compacta, which is less typical as the SN pars reticulata region is more affected [36]. DeFiglia and colleagues also

observed many shrunken and degenerating HTT-labelled neurons in cortical layers 5 and 6 at 2 weeks in mice that received an intra-striatal injection of an AAV1/8 vector expressing a truncated mHTT transcript (400 a.a with 100Q) [33].

Together these results suggest that viral vector-based modelling can reproduce many of the features of the human disease but artifacts in the disease model might also be introduced by depending on the viral vector type used and thus unexpected results may need to be interpreted cautiously.

More recently, lentiviral vectors optimised for astrocytic targeting by pseudotyping with the Mokola viral envelope and using the astrocyte-specific glial fibrillary protein (GFAP) promoter to drive transgene expression has been used to investigate the effect of expression of mHTT in astrocytes. [37] Reactive gliosis and decreased expression of glutamate transporters leading to altered glutamate uptake and neuronal dysfunction were found, consistent with that found in brain samples from HD disease subjects. This provides an alternative model for examining the contribution of mHTT-mediated astrocyte dysfunction to HD pathogenesis.

1.2.2 Mutant HTT transcripts and expression levels

Various N-terminal truncated *mHTT* transcripts have been expressed using the viral vectors as described above, with comparisons conducted relative to *HTT* fragments matched for size and linked to normal CAG repeat lengths (<30Q). Animals that receive control HTT vectors typically show diffuse neuronal HTT immunostaining in the striatum, and no evidence of intracellular inclusion formation or neuronal toxicity throughout the study.

Senut et al., [30] were the first to demonstrate that cumulative expression of expanded polyQ repeats throughout the life is not required to induce cell death but rather acute overexpression of polyQ is toxic to neurons *in vivo*. AAV2-mediated expression of expanded polyQ transcripts fused to green fluorescent protein was sufficient to induce the formation of intranuclear aggregates or large inclusion bodies as early as 5 days, with the majority of GFP-expressing neurons showing ubiquitinated aggregates by 12 days, leading to the degeneration of striatal neurons.

Several groups extended this work to characterising models based on expression of N-terminal truncated mHTT transcripts; de Almeida and colleagues conducted a comprehensive evaluation of the relationship between mHTT expression levels, polyglutamine repeat size (19, 44, 66 or 82 CAG) and protein length (N-terminal fragments consisting of the first 171, 853 or 1520 amino acids of human HTT). [31] Similarly, studies by deFiglia et al., and Franich et al., use constructs that fall with these ranges (the first 400 amino acids of HTT and 18 or 100Q)[33], (exon 1 of HTT and 20 or 70Q)[32]. In these studies, HD neuropathology at specific time-points ranging from 1-12 weeks were examined.

Nuclear HTT aggregates appeared as early as 1 week after viral vector injection, are ubiquitinated by 2 weeks [31] and progressively accumulate over the first 4 weeks. The size of the aggregates are dependent on mHTT expression levels, with lower levels associated with intranuclear and neuritic aggregates and dystrophic neurites, while larger intranuclear inclusions are found with higher transgene expression [33,31]. Evidence of neuronal loss can occur as early as 2 weeks in the immediate vicinity of the injection site, coinciding with the peak of nuclear inclusion accumulation. A loss of anti-HTT aggregate immunostaining then coincides with extensive neuronal degeneration and reactive gliosis from 5-12 weeks after injection

and motor impairment [32]. The time course of neuropathology is influenced by the specific transcripts expressed, with shorter mHTT fragments, longer CAG repeats and higher expression levels resulting in an earlier onset and more severe pathology[31].

Two of these models have been used successfully to demonstrate the efficacy of RNA interference-based therapies for HD. Therapeutic silencing of mHTT with siRNA or shRNA promoted neuronal survival as well as reduced the numbers of neuropil aggregates and size of inclusions [33,32], and prevented impairments in motor function as assessed by spontaneous forepaw usage.[32]

1.3 Non-human primate models of HD using viral vector-mediated approaches

Viral-vector mediated modeling approaches have been extended to applications to non-human primates. In a landmark study, Palfi et al. [38] injected lentiviral vectors expressing mHTT171-19Q or mHTT171-82Q into the dorsolateral putamen of macaque monkeys. Four injection sites were chosen covering the dosolateral aspect of the commissural and post-commisural putamen, a region known to be involved in dyskinesia in primates with unilateral excitoxic lesions [39]. Similar to the pathology observed in rats, mHTT171-82Q expression was associated with the formation of neuritic and nuclear ubiqutinated aggregates, loss of staining for the neuronal marker NeuN and astrogliosis at 9 weeks post-vector infusion. By 30 weeks, the size of EM48 HTT inclusions were smaller and atrophy of the putamen was clearly evident. When the vector was infused unilaterally, no changes in spontaneous behaviour were observed over the 9 week period but choreiform movements were induced by apomorphine injection. Bilateral infusions of vector led to display of a spectrum of movement deficits beginning at 16 weeks and continued for up to 30 weeks including hand, leg and head dyskinesia, leg dystonia, and even tics. Furthermore, behavioral

analysis of these animals showed that their neurological deficits progressed in a manner similar to the progression observed in HD patients.

The first transgenic monkey model of HD has recently been generated by Chan and colleagues [40] using a combination of transgenesis approaches and viral vector technology. Lentiviral vectors expressing exon 1 of the human *HTT* gene with 84 CAG repeats under the control of the human polyubiqutin-C promoter were injected into the pervitelline space of rhesus monkey oocytes. Five live newborns were delivered at full-term, with all transgenic monkeys carrying the transgenic mHTT genes but with variable repeat lengths ranging from 29-88 CAG. Three newborns carrying between two and four copies of mHTT developed severe symptoms, with two monkeys died shortly after birth, while the other survived for 1 month. Of the remaining monkeys, each of which caries a single copy of the mutated gene, one has only 29 CAG repeats and so shows no disease symptoms while the other has 83 CAG repeats and has developed low level deficits in movement coordination and involuntary movements such as chorea and dystonia which began 1 week after birth. HTT aggregates or inclusions were found in the striatum and cortex, with increased intensity of EM48 immunostaining correlating with high levels of mHTT with longer repeats. No obvious signs of neurodegeneration were found in striatum of the monkey that died shortly after birth.

The ongoing development and characterisation of these large animal models will be valuable resources for therapeutic screening of new HD therapies. These recapitulate the features of human disease, will be valuable in optimisation of delivery of cell and gene therapies, help understand relationship between mHTT expression levels, neuropathology and motor dysfunction.

1.4 Summary

These data show that viral vector-based rodent and primate models of HD can be a powerful complement to existing transgenic models of HD and can play an important role in the screening of new therapies for this devastating disease. Methods for generating a rat model of HD are described below.

2. Materials:

1. Viral vectors. We used a chimeric AAV1/2 vector in our study but other AAV serotypes including AAV5, AAV8, AAV9 that transduce large volumes of the rat or mouse striatum would also be suitable as well as the lentiviral vectors as described above and can be obtained through established vector core facilities or suppliers (e.g GeneDetect.com., Penn Vector Core, University of Pennsylvania). Specific N-terminal mHTT fragments as described above can be expressed; longer fragment sizes will be associated with slower rate of disease progression. (also see Note 1).
2. Animals. We typically use male Wistar or Sprague Dawley rats in the 230-300 g weight range.
3. Stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, CA).
4. Rotary drill. We use a micro-drill that was purchased from Fine Science Tools (USA) Inc. Foster City, CA. A similar item (catalog number 58610) can be purchased from Stoelting Co., Wood Dale, IL.
5. Hamilton syringes. 10uL 701N point style #2, catalog number 80300; Hamilton, Reno, NV.
6. Microinfusion pump. UltraMicroPump with Sys-Micro4 controller; World Precision Instruments, Sarasota, FL. See http://www.wpiinc.com for distributors

7. 0.9% (v/v) saline: Weigh 9 g of NaCl and add distilled water to a final volume of 1 L.
8. 4% (w/v) paraformaldehye (PFA) in 0.1 M phosphate buffer, pH 7.4: Make an 8% PFA solution by weighing 40 g PFA and dissolve in 500mL distilled water. Stir solution and heat to 60^{o}C in a fume hood. Add 1N NaOH in a dropwise manner using a pipette until the solution clears. Cool and filter to remove any particular matter. Dilute ½ with 0.2M phosphate buffer, pH 7.4. For 0.2M phosphate buffer, make a dibasic solution by dissolving 28.39g Na_2HPO_4 in distilled water and make up to final volume of 1L. Make a monobasic solution by dissolving 27.6g $NaH_2PO_4.2H_2O$ in water and make up to a final volume of 500mL. Add the monobasic to the dibasic solution until pH 7.4 is obtained.
9. 10, 20 and 30% (w/v) sucrose in 1x phosphate-buffered saline (PBS), pH 7.4: Dissolve 100g, 200g or 300g sucrose in 1 x PBS to a final volume of 1L. To make a 10xPBS stock solution, dissolve 80g NaCl, 2g KH_2PO_4, 2g KCL, 11.5g Na_2HPO_4 in water and make up to a final volume of 1L and adjust to pH 6.8 with HCl. Once diluted to 1xPBS, (1/10 with distilled water), this should be at pH 7.4.
10. Spontaneous forelimb use perspex cylinder: made in-house. Dimensions 20cm diameter x 30cm height
11. Automated video-tracking system: Noldus Ethovision XT. See http://www.noldus.com for distributors
12. 2 m long, 20 cm wide runway into an escape box. Made in-house.
13. Rotorod: TSE Rotarod Advanced. TSE Systems Inc., Chesterfield, MO. see www.tse-systems.com for distributors

3. Method

3.1 Stereotaxic Delivery of Vectors into the Rat Brain

This section describes our procedures for infusion of viral vectors into the striatum.

1. Animals are anaesthetised according to institutional guidelines and the animal positioned in a stereotaxic frame.
2. Make an incision through the scalp to expose the skull surface and identify bregma and lambda points. Adjust the incisor bar height such that bregma and lambda are at equal dorsoventral height (flat skull).
3. Coordinates for intra-striatal infusion of viral vector into the rat brain are determined with reference to the atlas of Paxinos and Watson (1986) [41] and are as follows: anterior-posterior (AP) +0.4 mm, mediolateral (ML) -3.0 mm, dorsoventral (DV) -5.5 mm, bregma = 0.
4. Make a small burr hole in the skull using a high-speed rotary drill at the appropriate AP-ML coordinates and lower the tip of a 10 µL Hamilton syringe with a 25 gauge needle under control of a microinfusion pump controller into the infusion site.
5. AAV vector (up to 3 µL) is infused into the striatum at an infusion rate of 70-100 nL/min. (see Note 2)
6. After completion of the infusion, the syringe is left in place for a further 5 min to allow diffusion of the vector before it is slowly withdrawn over a 5 min period and the scalp sutured.
7. Follow institutional guidelines for application of analgesia and monitoring recovery from surgery as required.

8. At specific time-points following vector infusion, rats or mice are transcardially perfused with 0.9% (v/v) saline followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (4% PFA).
9. Brains are fixed overnight in 4% PFA before cryoprotection by immersion in 10% sucrose in PBS. The following day, remove the 10% sucrose solution and replace with 20% sucrose in PBS. The brains will float to the top of the tube. Once the brains have sunk to the bottom of the tube (1-2 days), replace solution with the 30% sucrose in PBS. The brains are ready for cryosectioning once they have sunk to the bottom.

3.2 Behavioural Testing

Impairments in motor function can be assessed using specific motor function tests. We used the spontaneous exploratory forelimb use test to confirm the motor deficit in our rat HD model[32] but other tests as described below can also be used. (see Note 3).

3.2.1 Spontaneous exploratory forelimb use

Motor impairment following unilateral lesioning of the striatum can be assessed by spontaneous exploratory forelimb use. Rats are placed inside a transparent perspex cylinder (20cm diameter x 30cm height) and forelimb use during exploratory activity is recorded by video recording for 5 min. Two mirrors were placed behind the cylinder at an angle to enable recording of forelimb movements when the rat is facing away from the camera. The forelimb used for push-off and landing of vertical movements within the cylinder is counted by reviewing the vido footage in slow motion. Net ipsilateral forelimb use values are expressed as a percentage of total forelimb use.

3.2.2 Spontaneous Locomotor Activity in an Open Field

Spontaneous locomotor activity can be assessed by placing rats in a circular enclosure, 1.8 m in diameter divided into segments of equal area. Locomotion under dim lighting condition is video-recorded for 5 min and the number of lines (the boundary between each segment) crossed is manually quantified by the investigator. Monitoring of this type of activity can also be conducted by utilising automated video-tracking systems.

3.2.3 Footprint Test

The gait of rats can be analysed to detect deficits in locomotion. Rats are trained to walk along an enclosed 2 m long, 20 cm wide runway into an escape box. On the day of the trial, place the rats' hind- and forepaws in red and blue non-toxic dye, respectively (20% food colouring in glycerol), before releasing the rat onto a strip of paper on the floor of the runway and allowing the rat to walk into the escape box. The footprint patterns are assessed quantitatively by measurements of forepaw and hindpaw stride length, base width and forepaw/hindpaw footprint overlap. The mean values from each set of 6 steps are calculated.

3.2.4 Rotarod Performance

Rats' locomotor coordination is assessed on an accelerating rotarod. Rats are placed on the rotarod which accelerates from 4 to 40 rpm over 5 min. Rats are given two training sessions and one trial per day over 3 days when the latency to fall is recorded.

4. Notes

1. Our AAV1/2 HD rat model has a rapid onset of neuropathology with significant cell loss occurring by 2 weeks.[32] We found in subsequent studies that slight

differences in virus batches can shift the time course of neuropathology quite dramatically, which can create difficulties when using previous results as a basis for the design of studies aimed at testing new therapies. Similarly, deFiglia et al., [33] reported differences in extent of protection with siRNA against HTT in animals injected with two different batches of AAV vector. To increase the likelihood that the time-course of effects will be consistent between studies, we recommend generating a large batch of viral vector stock, conducting pilot studies in animals to characterise the time course of neuropathology obtained. The same vector stock can then used in subsequent studies.

2. Include appropriate controls such as vectors expressing the same mHTT fragment but with a normal (e.g <36) CAG repeat length. Performing unilateral infusions also allows comparisons between the injected and uninjected contralateral hemisphere as an internal control.
3. Prior to commencement of the tests, all animals should be pre-handled 5 min per day for 3-5 days by the investigator performing the tests to acclimatize them to being handled, preferably in the room where the tests will be performed.

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