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Review

Large Animal Models of Huntington's Disease: What We Have Learned and Where We Need to Go Next

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Abstract. Genetically modified rodent models of Huntington's disease (HD) have been especially valuable to our understanding of HD pathology and the mechanisms by which the mutant *HTT* gene alters physiology. However, due to inherent differences in genetics, neuroanatomy, neurocircuitry and neurophysiology, animal models do not always faithfully or fully recapitulate human disease features or adequately predict a clinical response to treatment. Therefore, conducting translational studies of candidate HD therapeutics only in a single species (i.e. mouse disease models) may not be sufficient. Large animal models of HD have been shown to be valuable to the HD research community and the expectation is that the need for translational studies that span rodent and large animal models will grow. Here, we review the large animal models of HD that have been created to date, with specific commentary on differences between the models, the strengths and disadvantages of each, and how we can advance useful models to study disease pathophysiology, biomarker development and evaluation of promising therapeutics.

Keywords: Minipigs, sheep, nonhuman primates, therapeutics

Since the cloning of the *HTT* gene in 1993 [1], there has been an explosion in the generation and use in HD research of genetically modified animal models that harbor part or all of the mutant *HTT* (m*HTT*) gene (reviewed in [2, 3]). Given their relative ease to engineer, propagate and use in a typical laboratory setting, the emphasis has been on using Huntington's disease (HD) rodent models. However, HD drug development has many examples where benefit was evident in mice but without a notable effect when tested in humans. Recent examples include a phosphodiesterase 10 inhibitor [4], and coenzyme Q10 [5], amongst others, reviewed in [6]. There are now candidate large-molecule HD therapeutics either in, or approaching, clinical trial, including antisense oligonucleotides, siRNAs, AAV-driven miRNAs, and gene editing, i.e., CRISPR-cas9 (reviewed in [7]). Extending translational testing of these large molecules, particularly to large animals to take advantage of their larger brains

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and anatomy closer to that of human (as compared with rodent), will better model CNS distribution and enable evaluation of local versus global effects on neurophysiology. Further, the longer life span (10–35 years) of the large animal species being developed into HD models will allow long-term (years rather than weeks or months in rodents) tracking of safety and efficacy.

Significant questions remain. Which large animal species are most suitable to model the complex neuropathological and behavioral sequelae of mHTT-mediated disease? Should the full *HTT* gene sequence be included or will fragments be acceptable? What is the most suitable CAG repeat size? Importantly, do we need to emphasize large animal models that recapitulate the now well characterized (thanks to observational studies PREDICT HD, TRACK-HD, TRACK-On-HD, and HD-YAS) long prodromal period in Huntington's disease gene expansion carriers (HDGECs), or those that rapidly develop overt disease signs that phenocopy manifest HD? Do we need both slower and faster progressing models or can we capture all required features in a single model? The answers to these questions have serious implications due to the expense and time commitment required to generate and characterize large animal models; only a select few well-positioned strategic attempts are feasible.

SHEEP MODELS

Sheep have recently been developed to study HD [8–11], and the sheep HD model [10] in particular is a useful addition to rodent models. Sheep have larger brains (∼120 g) than rodents (∼0.4 g) and non-human primates (NHPs) (∼85 g), with a prominent gyrated cortex and large neostriatum similar to human brains (∼1400 g). Compared to rodents, these brain anatomical similarities and longer lifespans (reviewed in [12]) also make sheep (and other large animals) appropriate candidates for longitudinal MRI and EEG studies. Sheep also have a metabolism and complex immune system more similar to humans, and are amenable to frequent blood and CSF collections (reviewed in [12, 13]). Sheep placidity renders them easy to manage, and they are relatively inexpensive to maintain compared to NHPs [11, 14].

However, there are disadvantages; sheep have a thick skull that complicates brain extraction (sometimes leading to tissue damage and degradation), and generating large groups for research studies can be challenging due to their longer gestation (152 days vs. 21 days for rodents) and smaller litters (1.5 animals per lambing). Q-fever, a rare zoonotic disease caused by the bacteria *Coxiella burnetii*, is also a risk in sheep flocks as it can cause abortions and still-births and can infect humans through reproductive tissue exposure and environmental contamination [15, 16]. However, overall sheep offer advantages that outweigh the disadvantages as HD models (Table 1).

An HD transgenic sheep model (OVT73) was created in 2010 [10] that does not exhibit many of the overt phenotypes observed in HD patients, but the existing data suggests that it may be a reasonable model of prodromal or early-stage HD. The 11.9 kb transgene fragment (Fig. 1) contained full length human *HTT* cDNA (67 exons) ligated to a 1.1 kb human genomic DNA immediately upstream of exon 1 that contains the promoter [10]. The 3' end of the construct contained a bovine growth hormone genomic exon 4-intron-exon 5 fragment that contains the polyadenylation signal. The CAG tract used in exon 1 was a pure stretch of 69 CAGs followed by a penultimate CAACAGCAACAG sequence, thus encoding 73 Qs. Consistent with HD phenotypes, the OVT73 sheep develop significant loss of cannabinoid receptor 1 (CB1) and dopamine-and cyclic AMP-regulated phosphoprotein (DARPP-32) within the globus pallidus [10, 17]. They also exhibit circadian-based behavioral disturbances [11]. Notably, the circadian abnormalities were linked to social grouping of the animals. Robust abnormalities emerged only in transgenic sheep when kept in flocks with other transgenic sheep; and not when mixed with normal sheep, whom are likely exerting social pressures on the transgenics resulting in a masking of the phenotype. Similarly, the authors note that sleep-wake abnormalities in HD patients are likely to be masked or dampened, possibly as a result of social pressures, and that such symptoms in HDGECs may precede overt symptoms by many years. Elevated levels of metabolites in the liver such as nicotinic acid, myristic acid and dodeconic acid, and an altered plasma metabolomic profile are also evident in the transgenic sheep [18]. Increased brain urea is evident in the striatum, along with early biomarkers of dysfunction in urea cycle and nitric oxide metabolism [8, 9, 18]. Importantly, OVT73 sheep express mHTT in the brain and develop mHTT aggregates and inclusions bodies in a neuroanatomical pattern consistent with HD [17]. mHTT is detectable in CSF and plasma, enabling translational studies that seek to non-invasively track the degree of mHTT lowering.

Table 1
Outline of advantages and disadvantages of using sheep as animal models for HD

Advantages	*Disadvantages*
Large brain & gyrated cortex	Thick skull
Prominent neostriatum	
Long lifespans [10+ years]	
Amenable to frequent blood & CSF collections	
Similar immune system to human	Sheep can acquire Q fever
Sheep cognitive tests have been developed	
Docile & easy to manage	
Inexpensive to maintain compared to NHP	Large space requirements
Transgenic HD model exists	
– OVT73	
– cDNA for full length human mutant *HTT*	– No genomic intronic or 3'UTR sequences
– 69 pure CAG repeats	– Sub-endogenous levels transgene expression
	– 2 copies of sheep *Htt* gene intact
– Models pre-symptomatic disease	
– useful for HTT lowering PK-PD-safety studies	
– Metabolomic, histopathological changes	– Lacks overt/ robust behavioral phenotype
– Modest motor/circadian phenotype	
– Sizable flock and infrastructure (Australia)	– Tg model not readily available outside of AUS
– Model access through CHDI	

The OVT73 sheep is the only large animal model available to investigate molecular therapies targeting anywhere along the full-length human *HTT* gene coding sequence, which has allowed investigators to validate engagement of an AAV9-encoded miRNA targeting the human *HTT* gene and its neuronal uptake within brain structures [19, 20]. These findings enabled evaluation of the safety, biodistribution and HTT-lowering activity of a molecular therapeutic in a large brain, leading to future clinical translation.

While some HD phenotypes are mimicked in OVT73 sheep, there are important gaps. These animals exhibit no dystonia, chorea, nor overt anatomical brain differences, and only mild or no neurological abnormalities (unpublished data and [8]), although this has only been partially analyzed out to 5 years old. It has been suggested that it may take > 5 years for OVT73 sheep to manifest the full pathology observed in humans, such as motor dysfunction, cognitive decline and weight loss [14], given that the CAG repeat size was a juvenile-onset pure CAG tract length of 69. The lack of a robust phenotype may also be partly due to the fact that a heterologous cDNA transgene construct was used to generate the model which may be responsible for sub-endogenous expression levels of the transgene-derived mRNA and protein [17]. Lack of *HTT* genomic sequences, including 3'UTR and introns, could prevent important HTT mRNA species to be expressed. Absence of intron 1 genomic DNA sequences in the transgene construct would prevent the expression of the splice read-through mHTT exon 1 -encoding mRNA, postulated to be an important species in HD pathophysiology [2, 21]. It is possible that a larger CAG repeat is required in sheep to generate pathology and overt motor (and possibly cognitive) signs in a shorter timeframe. Additionally, striatal disease in quadrupeds [22, 23] has not yet led to gait disturbances like those experienced by HD patients. To our knowledge, a relationship between movement disorders and extrapyramidal nuclear lesions in ungulates has not been published. Extrapyramidal basal nuclear lesions in ungulates, including the caudate, putamen, globus pallidus and substantia nigra, are rare in veterinary medicine and do not result in movement disorders or gait propulsion abnormalities. Disease signs associated with such lesions include propulsive activity (pacing, circling, head turning), uncontrolled involuntary muscle contractions, and spastic paresis of the lips, tongue and pharyngeal muscles [22, 23]. Movement disorders in nonhuman animals are best recapitulated in dogs with dyskinesia, but these diseases are usually breed dependent, inherited, and lesions are not within the basal ganglia (reviewed in [22, 24]). Consequently, we do not know whether (and if so, how) motor dysfunction might develop in a sheep HD model—it could well be different to the chorea seen in humans.

Minipig models

Of the various large animal species used as models in biomedical research, minipigs have a number of advantages and could be a species of choice for

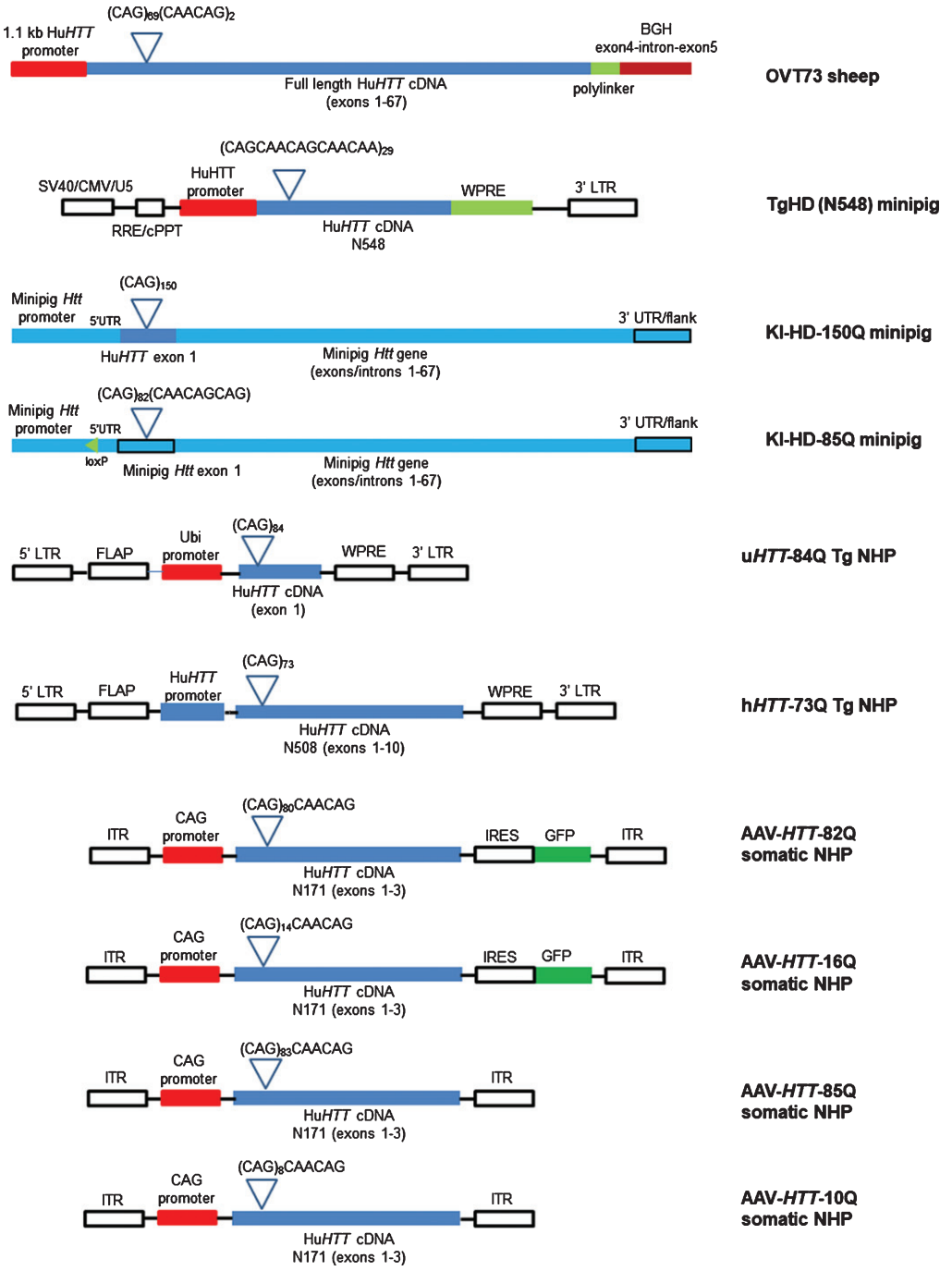

Fig. 1. Schematic representations of *HTT* gene structures in the HD sheep, minipig and NHP models. Gene structures were derived from: OVT73 transgenic sheep [10]; TgHD (N548) transgenic minipig [43]; KI-HD-150Q minipig [53]; KI-HD-85Q minipig (Exemplar Genetics personal communication and D. Howland; this article); transgenic NHP models: u*HTT*-84Q and h*HTT*-73Q [67]; and the somatic NHP AAV-*HTT*-82/85Q and AAV-*HTT*-16/10Q (J. Mcbride; this article) [73]. Lentiviral and AAV DNA vector elements are depicted in open white squares. BGH, bovine growth hormone; UTR, untranslated region; GFP, green fluorescence protein; Ubi (promoter), ubiquitin; CAG (promoter), cytomegalovirus early enhancer element/promoter-first exon-first intron of chicken B-actin gene/splice acceptor of rabbit B-globin gene; SV40/CMV/U5, simian virus 40, cytomegalovirus, unique 5; RRE/cPPT, rev response element, central polypurine tract; LTR, long terminal repeat; FLAP, a 3-stranded DNA structure; WPRE, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element; IRES, internal ribosome entry site; ITR, inverted terminal repeat.

Table 2
Outline of advantages and disadvantages of using minipigs as animal models for HD

Advantages	*Disadvantages*
Large gyrencephalic brain with similar neuroanatomy and blood supply to humans	Two layers of frontal bone with large inter-bone gap
Similar neurodevelopmental processes and comparable white matter ratio and degree of myelination to humans, striatum is prominent and divided into separate caudate and putamen	
Similar immune system to human	Susceptible to PERV (porcine endogenous retroviruses)
Long lifespans (10–20 years)	
Omnivores, digestive system similar to humans, body weight 80–110 kg	
Amenable to frequent blood & CSF collections	
Able to reproduce at 6 months	
Gestation only 4 months	
6–8 piglets per litter, thus relatively easy preparation of experimental groups for preclinical studies	
Relatively easy to maintain in controlled conditions in stables	Boars have to be stabled separately or castrated.
Inexpensive to maintain compared to NHP	
Minipigs can learn some cognitive and motoric tests	Minipigs are tetrapods and so gait and balance are dissimilar to those of humans. Minipigs do not have forearms and fine motor skills can be tested only by tongue test
Genetically modified models exist:	
1. TgHD (N-548)	
– Models long premanifest stage	– Slow phenotype progression; manifest symptoms late – CAG/CAA repeat structure
– Useful for HTT lowering PK-PD, safety studies	– two endogenous minipig *Htt* alleles intact
– Model access through CHDI/IAPG	
2. KI-HD-85Q (minipig *Htt*; 82 pure CAG repeats)	
– Model access through CHDI/IAPG	– 100% porcine *Htt* sequence
– Useful for HTT lowering PK-PD, safety studies	– Phenotype not well described
3. KI-HD-150Q (human *HTT* exon 1; 150 pure CAG repeats)	
– Models manifest disease	– Only 40% of F1 piglets survived longer than 5 months – Severe disease symptoms may be too rapid for interventional testing – Unknown ability to access model

HD research (Table 2). The main advantage minipigs have over pigs is that the former have an adult weight of 80–110 kg and do not grow too large. Minipigs have a relatively large, gyrencephalic brain with similar neuroanatomy and blood supply compared to humans, and have a comparable white matter ratio and degree of myelination whilst also undergoing similar neurodevelopmental processes [25]. Minipig brain size and neuroanatomy also make them suitable for neurosurgical procedures and non-invasive imaging methods similar to those used in human diagnostics [26]. Minipig physiology [25], and immune responses (around 80% similarity) [27–29], are more similar to those in humans when compared to lower vertebrates.

Minipigs have a relatively short gestation period (114 days), large litters (6–8) and mature sexually at 6 months [30], important advantages over NHPs and sheep both in terms of generating new models and establishing adequately powered experimental groups. Minipigs are easier to maintain in a controlled environment compared to herbivore sheep in pasture, although the latter is a more natural, less stressful environment. Boars must be housed in separate pens, but sows and castrated boars can be housed together. In addition, sheep are ruminants, which is a clear disadvantage for oral drug delivery and digestion. In contrast, minipigs are omnivores, and have a digestive system similar to humans [31].

A clear disadvantage relative to NHPs is the inability to assess fine motor movement in minipigs, particularly manual movement. While cognitive assays and behavioral assessments are not as well established for minipigs as for NHPs, they can learn tasks and use their snout to respond in cognitive tests. As with sheep, it is unclear how any motor dysfunction will manifest in minipigs expressing mHTT but, if present, it may be more difficult to measure

since quadrupeds can better compensate for any slight instability. Work to establish cognitive, motoric and behavioral tests in minipigs [32–35] and sheep [36, 37] is ongoing.

Before clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and TALEN (transcription activator-like effector nucleases) technology enabled much simpler genetic modification of endogenous pig genes [38–40], three attempts to generate HD transgenic minipigs have been reported. In 2001, a porcine m*Htt* cDNA (containing 75 CAG repeats) was microinjected into the pronucleus of fertilized eggs [41]. Two piglets with the transgene were born but mHTT was not expressed, probably due to transcriptional silencing or disruption of the transgene construct. In 2010, a HD transgenic minipig expressing the N-terminal segment of human m*HTT* with 105Q was created via somatic cell nuclear transfer (SCNT) (N208-105Q) [42]. All piglets with the transgene died within 53 hours after birth, presumably because of incomplete reprogramming during SCNT. In 2009, a transgenic HD minipig (TgHD N548) founder was born at the PIGMOD Center in Libechov, Czech Republic, after microinjection of a lentiviral vector encoding the N-terminal part of human m*HTT* (N548-124Q) under the control of the human *HTT* promoter (Fig. 1), into porcine embryos [43]. The mHTT, at both mRNA and protein level, was detected in all tissues with the highest level in the brain and testes [44, 45]. Mutant HTT protein was also detectable and quantifiable in blood and CSF [46], making the model very useful in investigating of the effects of candidate therapeutics on HTT lowering biomarkers. Biochemical evidence of mHTT aggregation was evident in the brain, but there were no discernable signs of macroscopically visual mHTT inclusions in the CNS [43]. Fluorescent *in situ* hybridization (FISH) and genomic analysis confirmed the incorporation of one copy of the mHTT transgene into the noncoding sequence of the first porcine chromosome (1q24-q25) without interrupting any coding sequence in the minipig genome [44]. Currently, the PIGMOD Center breeds TgHD (N548) minipigs and their wild type (WT) siblings with identical genetic background, which are subject to phenotypic evaluation as well as sponsored preclinical studies of HTT lowering interventions.

The HD phenotype in this model progresses gradually with age compared to age-matched WT minipigs. A severe reproductive phenotype precedes the gradual neurodegeneration, and is detected around the age of 13 months [44] with low count, reduced motility, low mitochondrial energy-generating system and respiratory parameters in TgHD (N548) boars' spermatozoa [47] and decreased phosphodiesterase (PDE) concentration (24 months) in testicular parenchyma [48]. The neurodegenerative phenotype starts gradually with reduction of DARPP-32, an integrator of neurotransmission, at 16 months in the neostriatum [43] and remains downregulated until at least the age of 70 months [45]. Gradual mHTT protein fragmentation, activation of microglia, and demyelination of white matter [45], together with a significant decrease of total creatine was detected at 24 months [48]. Functional locomotor decline together with genotype-specific effects on mitochondrial DNA (mtDNA) damage, mtDNA copy number, and markers of a metabolic alteration that manifests in progressive neuropathology were detected at 48 months [49]. Axonal inclusions together with age-dependent cellular degeneration was detected in TgHD (N548) minipigs at 60–70 months of age (unpublished), followed by a slowly progressive motor, cognitive and behavioral phenotype, and gender-specific loss of body mass index manifesting at 72–96 months [50].

The TgHD (N548) minipigs express both endogenous alleles encoding wild-type *Htt* (wt*Htt*) in addition to the mutant transgene, which might postpone the phenotype progression, since the loss or reduction of normal HTT function may play an important role in HD progression [51]. Also, the glutamine-encoding sequence in this TgHD (N548) minipig model is comprised of a repeating pentameric series of CAG and CAA triplets (CAGCAACAGCAACAA) rather than a pure CAG tract (Fig. 1), which was designed for better stability of the construct when generating this model in 2008 [43]. However, the mixed CAG-CAA tract could attenuate development of robust phenotypes since no CAG somatic instability [52] is evident. It may be possible that mutant HTT mRNA with a CAG-CAA mix sequence behaves differently than a pure CAG-encoding mRNA in terms of RNA-related toxicities, and protein products resulting from RAN translation.

In a collaboration between uniQure and the PIGMOD Center, the TgHD (N548) minipig model has been used to preclinically evaluate intracranial AAV5-miHTT administration, showing widespread vector distribution and considerable HTT lowering in brain and CSF, an important translational biomarker [46]. Based on these results and preliminary data of ongoing longitudinal experiments, the Food and Drug

Administration and the European Medicines Agency approved this approach for a Phase I/II clinical trial that started in June 2020, the first time an HD large animal model has enabled regulatory approval of an HTT-lowering therapeutic.

A knock-in (KI) HD minipig model has been generated at Exemplar Genetics and is currently bred at the PIGMOD Center. AAV-mediated homologous recombination in minipig fibroblasts followed by SCNT was used to expand the CAG tract to 82 pure repeats, followed by a CAACAGCAG sequence, resulting in 85Q in the porcine *Htt* gene (Fig. 1). Two KI-HD-85Q founders gave birth to 50 F1 piglets expressing porcine mHTT and wild-type HTT in equal amounts; they exhibit uncoordinated hind limb movement and show atypical inactive behavior, and some show increased anxiety-like behavior. These animals express full length mHTT in all tissues examined, including throughout the brain, and mHTT is also quantifiable in blood and CSF (unpublished observations). More time is needed to describe the gradual manifestation of phenotype progression in this model but it is already being used by companies investigating a PK-PD-biomarker relationship after central administration of HTT-lowering therapies.

The CRISPR/Cas9 system could be used to generate porcine HD KI models. In 2018, a KI-HD-150Q porcine model was generated using CRISPR-cas9 [53] to replace the porcine *Htt* exon 1 with the respective human exon containing a 150-CAG repeat (Fig. 1) in fetal fibroblast cells, followed by SCNT. However, 40% of F1 piglets died before the age of 4 months with phenotypes that include motor dysfunction, respiratory difficulties, loss of medium spiny neurons, caudate atrophy, and somatic and germline CAG instability. Experiments to measure the effect of candidate HTT-lowering therapeutics on manifest disease would be valuable in this model, but the phenotype might be developing too early. Full implementation of the KI-HD-150Q model will require more extensive phenotyping in F1, F2 and further generations to minimize any confounds that are not a consequence of mHTT expression, such as developmental abnormalities common in SCNT/cloning [54].

Engineering large animals that harbor the entire human *HTT* gene (full exons, introns, 5' and 3' UTRs) and that accurately express the full repertoire of HTT isoforms including mRNAs and protein that arise from readthrough exon 1 mis-splicing [21], alternative splicing, RAN translation and proteolytic fragmentation would be valuable to HD research. Such a model would be useful to drug discovery programs targeting any part of the human *HTT* gene sequence.

Nonhuman primate models

Before the causative *HTT* gene mutation was discovered, non-genetically modified animal models dominated the field; the earliest large animal models of HD were created by lesioning the striatum of rhesus and cynomolgus macaques using various neurotoxins. Striatal administration of ibotenic acid, quinolinic acid, kainic acid and the mitochondrial inhibitor, 3-nitropropionic acid lead to a dramatic loss of enkephalin- and dynorphin- positive striatal neurons and reactive gliosis [55–59]. Additionally, these neurotoxins induced behavioral outcomes such as chorea and apomorphine-induced dystonia as well as cognitive impairment including reduced performance on the object retrieval detour task of response inhibition. However, hallmark striatal mHTT protein aggregates were absent in these early neurotoxin-based models. (For a review of the HD neurotoxin-based models in rodents and NHPs, see [60]). A more recent study [61] reported that in addition to spontaneous dyskinesias and increased perseverative behaviors on a set-shifting task, quinolinic acid-lesioned macaques also showed deficits in glucose metabolism and D2 receptor density in the lesioned putamen, as measured by positron emission tomography (PET), with a concomitant loss of neurons in the striatum and dorsolateral prefrontal cortex [61]. While the neurotoxin-based models do not replicate the genetic root cause of HD, they do recapitulate several key cardinal features of the disease.

Most of the toxin-based models fell out of favor with the HD research community after the *HTT* gene mutation was identified [1], and subsequent NHP models have largely been created by viral-mediated delivery of a fragment of the human m*HTT* gene or via the development of transgenic HD macaques, both bearing *HTT* genes with expanded CAG repeats that encode mHTT proteins with elongated polyglutamine tracts (Q) at the N terminus. The first viral-mediated macaque model of HD was created by injecting the dorsolateral, sensorimotor area of the putamen with a lentivirus expressing the N171 fragment of *HTT* containing 82 CAG repeats (LV-*HTT*82Q), leading to progressive, spontaneous dyskinesia of the legs, arms, and trunk out to 30 weeks post-surgery [62]. At necropsy, brain tissue revealed $EM48^{+}$ mHTT inclusion formation, loss of the neuronal marker NeuN, and astrocytosis in the local area of injection.

Recent studies by the McBride laboratory at the Oregon National Primate Research Center have expanded on these important initial efforts. An adeno-associated virus (AAV) was chosen over lentivirus due to its safety profile (non-integrating; remains episomal), its ability to diffuse farther from the site of infusion and the existence of different AAV capsid serotypes with altered properties, including differential cell transduction. Macaques were injected with AAV serotype 1 expressing the N171 *HTT* fragment containing 82 CAG repeats (AAV1-*HTT*82Q) (Fig. 1) into the head of the caudate nucleus and the anterior and posterior regions of the putamen in an attempt to model both the cognitive and motor dysfunctions in HD. Compared to a control cohort injected with AAV1-*HTT*16Q, AAV1-*HTT*82Q treated animals developed a progressive motor phenotype over 40 weeks including mild chorea and dystonia that worsened upon apomorphine administration. Progressive worsening of fine motor skills, as measured using the Lifesaver Task, and a progressive decline of spatial working memory, using the 3-Choice Spatial Delayed Response Task, was also evident. Along with behavioral decline, transcriptional analysis on post-mortem tissue samples taken from both the caudate and putamen showed dysregulation of several genes in AAV1-*HTT*82Q treated animals compared to controls. The most significantly impaired pathways were neuronal signaling, amyloid processing, oxidative stress response, and mitochondrial dysfunction, corroborating findings from HDGEC samples and mouse models [63]. Immunohistochemical analyses showed loss of NeuN immunoreactivity, astrocytosis, microgliosis and mHTT inclusion formation in the caudate and putamen as well as the globus pallidus and substantia nigra, pars compacta to a lesser degree (manuscript in preparation). Because mHTT DNA fragments are typically driven from viral vectors with strong promoters such as CAG and CMV, it is possible that the phenotypes seen in these viral vector-based models could be due to the expression of supraphysiological levels of mHTT fragments, which is different than that of HD patients with endogenous levels of full length mHTT.

The first transgenic macaque model of HD was created at the Yerkes National Primate Research Center [64]. This group used lentiviral vectors to deliver a transgene fragment of the human *HTT* gene (exon 1) bearing 84 CAG repeats, driven from the human polyubiquitin C promoter (named u*HTT*-84Q; Fig. 1), to mature oocytes. Oocytes were fertilized and embryos were then implanted into surrogate female macaques. Of five newborns born, named rHD1-rHD5, rHD3-5 were euthanized soon after birth due to severe motor impairment (dystonia and chorea), including respiratory difficulties. It was determined that these animals contained multiple integration sites and expressed higher copy numbers of m*HTT* transgenes, carrying variable CAG repeat lengths at high levels, which correlated with their severe behavioral phenotypes. Post-mortem analysis showed robust nuclear and cytoplasmic mHTT aggregates throughout the brain, as well as in various peripheral tissues. rHD1 was followed longitudinally and showed a reduced striatal and hippocampal volume compared to controls, as well as impairment on hippocampal dependent memory tasks [65]. To expand on this work, a second cohort of transgenic animals (rHD6–8) expressing exons 1–10 of the human *HTT* gene bearing 67–73 CAG repeats, driven from the human *HTT* promoter were established [66] (named h*HTT*-73Q; Fig. 1) and monitored, along with controls, longitudinally out to 5 years of age. A large number of studies demonstrated that these transgenic HD macaques had elevated gene dysregulation in peripheral blood samples [67] and progressive decline in motor behavior (facial chorea, dystonia and seizures) and in cognitive function (perseverative errors on the ORDT) [66]. Temperament changes included irritability and anxiety-like behavior along with elevated levels of cortisol and pro-inflammatory cytokines [68]. Moreover, magnetic resonance imaging (MRI) studies in the transgenic HD monkeys demonstrated reduced caudate and putamen gray matter volumes and increased lateral ventricle volumes compared to controls [66]. Similarly, diffusion tensor imaging (DTI) analysis suggested microstructural changes in several white matter tracts throughout the brain [69]. Cell counts in two animals verified a reduction of both projection neurons and interneurons in the caudate and putamen, with elevated astrocyte number, that was *HTT* fragment length dependent [70]. Additionally, these two animals showed varying levels of mHTT aggregates.

Compared with the other large animal (minipigs and sheep) HD models, the various NHP models have more closely recapitulated many of the HD phenotypes evident in HDGECs, but there is still need for improvement (Table 3). One major downside to the transgenic HD macaque model [64, 65] has been the low number of animals that have been created to date beyond the initial founders and few F1 progeny, which has limited progress towards further charac-

Table 3
Outline of advantages and disadvantages of using NHPs as animal models for HD

Advantages	*Disadvantages*
Neuroanatomy, neurocircuitry and genetics are very similar to humans; long lifespan (∼35 years).	Long gestation (6 months), typically with singleton births.
Gyrencephalic brains with large cortices, striata – large, divided into separate caudate + putamen.	CAG repeat length in macaques is ∼10 compared to ∼35 in human patients.
Similar developmental stages and social structure to humans.	Expensive to generate, house, and maintain, including special housing and veterinary staff.
Highly visual and use facial expressions to communicate socio-emotional states	Macaques are quadrupeds and so gate and balance are dissimilar to those of humans.
Similar endocrine function to humans (monthly menstrual cycles and 24-hour hormone cycles).	Training macaques on complex tasks can be timely and dependent on individual temperament variability.
Can learn complex motor and cognitive tasks similar to those used evaluate in human patients.	Transgenic and somatic models express short fragments of m*HTT,* limiting the binding real estate of HTT-modifying therapeutics.
Macaques have forearms and digits that allow for testing of fine motor skills.	The majority of HD macaque models have only been generated in limited numbers.
HD macaques show progressive development of symptoms that mirror those seen in HDGECs.	mHTT in AAV somatic models is only expressed in specific brain regions, versus throughout the entire body.
Primary outcome measures used in clinical trials, including the motor UHDRS and volumetric changes via MRI, are affected in most NHP models	In somatic and transgenic models, NHPs express 2 copies of endogenous HTT, not replicating the genetics of human HD.
Gene dysregulation, cell loss, gliosis, regional brain atrophy and inclusion formation evident in HD NHP models.	Rhesus macaques carry zoonotics that are harmful if transmitted to humans
Somatic models allow for freedom in model design: viral serotype, viral promoter and dose	Neither transgenic nor viral-mediated models have been used to date to screen therapeutics for HD.
The majority of HD macaque models to date have been created and maintained in the US, making them accessible via collaboration (OHSU for AAV somatic NHPs and Emory University for transgenic NHPs).	Somatic models have only been generated in adult macaques, which do not accurately model human HD wherein mHTT is expressed from birth.

terization and candidate therapeutic evaluation. The biggest drawback in the viral-engineered models is that mHTT expression is primarily limited to the region of injection (to date putamen or a combination of the caudate and putamen), whereas in HD, there is clear pathology outside of the striatum. Vectors that can deliver a m*HTT* gene fragment to the striatum, in addition to all of the extra-striatal brain regions heavily affected in HD, would be a significant advance. AAVs with strong retrograde functionality, such as AAV2.HBKO and AAV2.retro, have recently been engineered and assessed in rodent and NHP brains [71–73], and they transduce injection sites - but are also transported to other afferent brain regions. McBride and colleagues recently showed that AAV2.retro-mediated delivery of *HTT*85Q into the adult rhesus macaque caudate and putamen leads to mHTT expression and aggregate formation in both of these brain regions as well as profound retrograde transport, with mHTT expression and aggregate formation in dozens of cortical and sub-cortical regions with known afferent projections to the striatum [73]. By inducing hallmark mHTT-mediated neuropathology throughout cognitive, motor and limbic circuits, this serotype is a promising delivery tool for better modeling of HD, versus targeting the striatum alone. A clear advantage of viral vector-based models compared to transgenic and KI models is that large numbers of animals can be generated relatively quickly. There is also more freedom in model design including the ability to assess different promoters, *HTT* gene fragment lengths, CAG repeat lengths, AAV serotypes or even combinations of serotypes in a shorter timeframe. To that end, evaluation of a cohort of 18 male and female rhesus macaques that have been injected into the caudate and putamen with a 1:1 mixture of AAV2 and AAV2.retro expressing *HTT*85Q or *HTT*10Q, or buffer alone, is in progress. Animals are being characterized out to 18 months post-surgery using a variety of imaging modalities (MRI, DTI, RSfMRI, and PET) as well as with complex motor, cognitive and temperament assays. CSF and serum samples are being evaluated for protein signatures of disease, including mHTT, and neurofilament light chain (NFL). As part of this work, a multimodal rhesus macaque brain atlas for use with MRI, DTI, RS fMRI and PET imaging has been generated, that will be published as an open resource for

the research community, for use in both modeling and therapeutic studies.

Another hurdle to overcome with the somatic and transgenic models is the limited packaging capacity of viral vectors used to deliver m*HTT* transgenes. Lentivirus has a packaging capacity of approximately 9 kB and AAV around 4.5 kB, thereby limiting the length of the *HTT* gene that can be packaged with these current vectors. Since a major goal of creating these models is to screen viable therapeutics, including HTT lowering therapeutics, reducing the potential real estate for targeting HTT-lowering agents is disadvantageous. For example, the lentiviral- and AAV-based models created to date contained the N171 fragment of *HTT* (exons 1–3) and the transgenic HD monkeys expressed either the N67 (exon 1) or N508 (exons 1–10) fragments of *HTT* (Fig. 1), limiting evaluation of microRNAs or antisense oligonucleotides that target sequences outside of these regions. Therapies that directly target the CAG repeat in exon 1, such as zinc finger repressors or CRISPR/Cas9, could be tested in each of these models.

With the recent advancement of gene editing technologies like CRISPR/Cas9 systems and TALENs, the creation of gene-edited KI NHP HD models, in macaques and marmosets, is now being pursued by multiple investigators (see Table 4). Although KI approaches generated several mouse lines with varying mutation types [2], these rodents do not fully recapitulate the pathologies and disease progression observed in humans. While transgenic approaches can be used in animal models that more closely follow human HD progression and phenotypes, the random insertion of the *HTT* gene or multiple copies into the genome may have unanticipated secondary effects. Using CRISPR/Cas9 or TALENs and a DNA template, it is now possible to directly deliver the desired gene variant of interest into the *HTT* locus [74]. In the presence of a DNA template, double-strand DNA breaks introduced by Cas9 can be repaired by homology-directed repair (HDR) whereby the template is used to specifically introduce an exact DNA sequence [75]. Moreover, the ability for CRISPR-based approaches to modify a target gene of interest was demonstrated in cynomolgus macaques, wherein green fluorescent protein (GFP) was inserted into exon 5 of the POU5F1 gene after the endogenous stop codon [76]. Collectively, these studies show that germline engineering of the *HTT* locus to generate NHP HD models is feasible.

While gene editing techniques have considerable potential to precisely deliver a defined number of CAG repeats into the *HTT* locus, some obstacles currently limit their use. At present, the efficiency of HDR in primate embryos is unclear. Results from human studies have revealed a wide range of HDR efficiencies (10–50%) [77, 78]. Off-target editing is also a concern, but the extent to which it occurs remains to be fully defined. In the cynomolgus macaque with the GFP-POU5F1 gene, whole-genome sequencing of the founder animal revealed minimal off-target edits [76]. Off-target editing rates are likely gene-dependent, which will necessitate a thorough characterization of off-target events in NHPs that are created by germline *HTT* editing. Other challenges in creating germline NHP HD models relate to logistical and infrastructure issues. Due to the length of gestation, the time and resources needed to create and care for the animals are substantial. However, based on the shared genetics and physiology of NHPs with humans, creating such models is needed to develop the next generation of therapeutics, which also includes the development of gene editing methods and delivery systems to restore normal HTT function.

Taken together these neurotoxin, viral vector and transgene-based NHP models have demonstrated success in recreating several of the neuropathological and neurological signatures of human HD. Interestingly, many of the cardinal features of HD in human reported in the NHP models do not yet seem to be recapitulated in the existing ungulate HD models (minipigs and sheep) including oral dyskinesias, forelimb chorea, hindlimb dystonia, aberrant posture, bradykinesia, perseveration, irritability, anxiety, aggression and spatial working memory decline. Of course, *Homo sapiens* and macaques share a relatively recent common ancestor (25 million years ago), as compared to ungulates, and share more similar motor, cognitive and limbic brain circuitry mediating some of these primate-specific behaviors [79]. Moreover, the neurotoxin, viral vector-based and transgenic NHP models have all shown a reduction in striatal volume, as measured via MRI. As motor dysfunction measured via the unified Huntington's disease rating scale (UHDRS) and striatal atrophy measured via MRI are two primary outcome measures commonly used in HD clinical trials, this places the NHP models as favorable candidates for pre-clinical therapeutic evaluation using similar outcome measures. As the biofluid biomarkers (mHTT, NFL) are also showing great promise as useful outcome measures in clinical trials, it will be important to establish these in the NHP models as well, as

Table 4
Summary of current efforts for development and use of large HD animal models

Model/Type	PI/Institute	Creation Technology	Species	Status	References
OVT73 Transgenic m*HTT* sheep	Snell (Auckland U.)	Embryo DNA microinjection/transgenesis	Merino sheep	Model delivered, phenotyping	[10, 17]
OVT73 Transgenic m*HTT* sheep phenotyping and HTT lowering	Aronin/Gray-Edwards (UMASS)		Merino sheep	HTT lowering and biomarker testing	[19, 20]
OVT73 Transgenic m*HTT* sheep phenotyping	Morton (Cambridge U.)		Merino sheep	Biomarker discovery - cognition and EEG	[11, 18, 36]
OVT73 Transgenic m*HTT* sheep phenotyping + HTT lowering	BioMarin		Merino sheep	Biomarker discovery and HTT lowering	Personal communication Sundeep Chandra, BioMarin
Knock-in m*Htt* sheep	Snell (Auckland U.)	Gene editing	Texel/dorset sheep	Model creation	Personal communication Russ Snell, Auckland U.
Transgenic m*HTT* minipig (TgHD) (N548)	Motlik/Ellederova (IAPG)	Lentiviral-mediated transgenesis	Libechov minipig	Model delivered, breeding, baseline phenotyping	[43]
Transgenic m*HTT* minipig: HTT lowering (TgHD) (N548)	Konstatinova (uniQure/IAPG)		Libechov minipig	Biomarker discovery and HTT lowering	[46]
KI-HD-85Q Knock-in m*Htt* minipig	Exemplar Genetics	AAV-mediated homologous recombination and SCNT	Libechov minipig	Model delivered	D. Howland, CHDI
KI-HD-85Q Knock-in m*Htt* minipig: breeding/phenotyping	Motlik/Ellederova (IAPG)		Libechov minipig	Cohort expansion and baseline phenotyping	D. Howland, CHDI
KI-HD-150Q Knock-in m*Htt* minipig	Li (Emory U.)	CRISPR gene editing/SCNT	Chinese minipig	Baseline phenotyping	[53]
AAV-m*HTT* somatic models	Mcbride (OHSU)	Somatic viral transduction	Rhesus macaque	Model creation, phenotyping, biofluid and imaging biomarker discovery	[73], this article
Transgenic m*HTT* (exon1 and N512)	Chan (Emory)	Lentiviral-mediated transgenesis	Rhesus macaque	Phenotyping and imaging biomarker discovery	[64–70]
Knock-in m*HTT* NHP	Feng (MIT)	Gene editing	Marmoset	Model creation	Personal communication Guoping Feng, MIT
Knock-in m*HTT* NHP	Hennebold (OHSU)	Gene editing	Rhesus macaque	Model creation	Personal communication Jon Hennebold, OHSU
AAV-m*HTT* homologous independent targeted integration	Okano (Keio U.)	Gene editing	Marmoset	Model creation	Personal communication Hideyuki Okano, Keio U.

another outcome measure that can be used to evaluate promising therapeutics.

Conclusions

The past decade has seen dramatic progress in the development of large animal models of HD in sheep, minipigs, and macaques. Investigations to create and characterize these animals have been immense, often requiring large collaborative scientific teams specializing in genetics, reproductive biology, complex behavior, multimodal imaging, neurosurgery and neuropathology. As these models are further characterized and refined they should be used in tandem with the rodent models that have been used so prevalently in HD research. Choosing which large animal model to use will depend heavily on the genetics, and the neuropathological and/or behavioral phenotypes under evaluation (see Table 4). There has been substantial progress and some real wins using large animal models in HD that have helped guide therapeutic candidates to clinical evaluation. This includes the use of non-genetically modified NHPs, used in late-stage drug development to evaluate biodistribution, pharmacodynamic (HTT lowering) and safety profiles. Extending these types of studies to large HD animals has begun and will allow for more comprehensive testing to include effects of candidate therapies on mHTT, disease-relevant phenotypes and biomarkers and importantly, long term safety in animals that have been exposed to mHTT for their entire life. However, there remain deficiencies in the repertoire of large animals models for HD that are currently available. We need models that cover the entire premanifest to manifest disease spectrum; and that have the entire human *HTT* gene sequence including introns, 5' and 3' UTRs, and a pure CAG tract expansion. This will allow investigation of a large spectrum of human *HTT* gene sequence-targeting therapeutics as such models incorporate all aspects of mHTT isoform expression—including the mHTT exon 1-encoding transcript [80], alternatively spliced mRNAs, RAN translation products and protein proteolytic fragments.

From what we have recently learned, higher CAG-repeat lengths (c. 110–150) may be needed to consistently produce overt disease, especially in minipigs and sheep. This CAG-length/overt-disease threshold may be different depending on both the species and specific *HTT* DNA construct used, but such long CAG-repeat lengths are only rarely found in juvenile-onset HD patients, raising the possibility that such models do not actually recapitulate adult-onset HD. Conversely, with an adult-onset CAG-repeat length (c. 40–50) the model animal may not develop robust signs of disease within a 10–20 year lifespan, nor in an even shorter timescale amenable to research. The decisions to introduce CAG-repeat lengths of 82 (85Q) in the KI minipig, 69 (73Q) in the transgenic sheep, and 73–85 in the NHPs were based on tract sizes considered reasonably likely to elicit prodromal/premanifest phases followed by neurodegeneration and overt behavioral phenotypes. It remains to be seen whether the KI-HD-85Q minipig or the OVT73 transgenic sheep fulfill this prediction, but frank neurodegeneration and overt phenotypes have not yet been reported. While there is evidence of a manifest phenotype in the somatic AAV1-HTT82Q and AAV2.retro-HTT85Q NHP models, this could be partially driven by the strong promoters that overexpress mHTT.

The extant large animal models should be used and further characterized; the TgHD (N548) minipig, the KI-HD-85Q minipig, and the transgenic (OVT73) HD sheep could be useful in modeling the prodromal and premanifest phases, and have already been useful in biomarker and safety studies. The KI-HD-150Q minipig and the AAV1-HTT82Q and AAV2.retroHTT85Q somatic NHPs may be used to model certain aspects of premanifest and manifest disease. The transgenic and somatic NHPs could be used to evaluate therapeutics that aim to slow progression of the hallmark motor (including chorea, dyskinesia and dystonia), cognitive (including frontal and temporal based working memory decline) and psychological (including impulsivity, aggression and anxiety) signs of disease.

Species selection in future large animal modeling should be based on factors such as brain size and complexity, generation time, phenotypes, and times to phenotype onset that investigators need to be evident. For instance, if models are required to investigate cardinal HD neuropathology, NHPs would be the first choice, but the expense and time to sexual maturity ($\sim$ 4 years) may be a limiting factor. The AAV-m*HTT* somatic NHP models will likely mitigate the timing issue and appropriately powered cohorts can be generated for model characterization, biomarker discovery and therapeutic evaluation. Attempts to generate germline HD KI NHP models are now underway (Table 4) and look promising, but many hurdles remain to determine whether germline transmission is feasible, sufficient progeny can be generated, and how disease phenotypes will present.

NHPs, minipigs, and sheep all remain viable options as large animals to model HD, and species choice should be considered on their strengths and weaknesses. Minipigs have the largest litters with the shortest gestation time, but sheep models have faithfully reproduced other human neurological conditions (CLN1, Tay-Sachs, Cystic fibrosis) [80–83] and are amenable to neurological and behavioral testing. The extant KI-HD-85Q and TgHD (N548) minipigs and transgenic (OVT73) sheep are certainly appropriate for early-stage PK-PD-safety and possibly biomarker testing, even without a fully manifest behavioral phenotype. CRISPR-mediated gene editing has been achieved in both minipigs [84, 85] and sheep [83, 86], paving the way for the generation of fully humanized (entire human m*HTT* genomic sequence) models and work is underway.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare

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