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Review

Large Genetic Animal Models of Huntington's Disease

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Abstract. The dominant nature of the Huntington's disease gene mutation has allowed genetic models to be developed in multiple species, with the mutation causing an abnormal neurological phenotype in all animals in which it is expressed. Many different rodent models have been generated. The most widely used of these, the transgenic R6/2 mouse, carries the mutation in a fragment of the human huntingtin gene and has a rapidly progressive and fatal neurological phenotype with many relevant pathological changes. Nevertheless, their rapid decline has been frequently questioned in the context of a disease that takes years to manifest in humans, and strenuous efforts have been made to make rodent models that are genetically more 'relevant' to the human condition, including full length huntingtin gene transgenic and knock-in mice. While there is no doubt that we have learned, and continue to learn much from rodent models, their usefulness is limited by two species constraints. First, the brains of rodents differ significantly from humans in both their small size and their neuroanatomical organization. Second, rodents have much shorter lifespans than humans. Here, we review new approaches taken to these challenges in the development of models of Huntington's disease in large brained, long-lived animals. We discuss the need for such models, and how they might be used to fill specific niches in preclinical Huntington's disease research, particularly in testing gene-based therapeutics. We discuss the advantages and disadvantages of animals in which the prodromal period of disease extends over a long time span. We suggest that there is considerable 'value added' for large animal models in preclinical Huntington's disease research.

Keywords: CAG repeat, polyglutamine, sheep, non-human primate, monkey, minipig, neurodegeneration, sleep, circadian

INTRODUCTION

Neurological diseases such as Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia and depression are complex in their manifestation, with symptoms that encompass motor, cognitive and psychiatric domains. Animal models of such diseases are invaluable tools that can be used for both understanding the mechanisms underlying pathology of neurological disorders, and also testing potential therapies. But the insights gained from different animal models vary markedly, depending on the model. Many neurodegenerative disorders, including HD, are uniquely human conditions. Therefore, we should not expect that any genetically modified animal used to model HD can recapitulate fully the pathophysiology and progression of the human disease.

HD is caused by a single dominant gene mutation that is an expanded CAG repeat in the *HTT* gene [1]. Although it is a rare disease, the fact that it is a single gene disorder makes it a 'paradigm' disease. That is, if we can develop a strategy for successfully treating HD, then there is an increased likelihood that we will be able to develop treatments for more genetically complex diseases such as AD. In 1993 when the HD gene was cloned, the expectation was, both within and outside the HD field that with the discovery of the

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gene would come immediate insight into the mechanisms underlying HD. But, not only was the nature of the mutation causing HD novel, the gene encoded for a protein that was hitherto unknown [1]. Furthermore, the expression of the huntingtin gene (*HTT*) gene and protein (HTT) was not specific to the striatum, the site of primary pathology in HD. In fact, it is not neuron-specific, or even brain-specific [2, 3]. A further twist comes from recent evidence suggesting that, although there is considerable evidence for toxicity arising from a gain of function of mutant HTT (for discussion, see [4, 5]), the gene product may not be the only toxic entity in HD. It seems increasingly likely that a contribution to the toxicity might arise from toxic mRNA or via aberrant transcription of the gene ([6, 7]; for reviews, see [8, 9]). Nevertheless, our understanding of the pathophysiology and clinical features of HD has grown steadily since 1993 ([10, 11] for a selection of reviews, see [12–22]). Importantly, although as yet there is no disease-modifying therapy these available [23, 24], there are several promising novel therapeutic strategies that are currently being developed [25–27]. Thus, while any expectations of a therapeutic 'quick fix' have been dashed, our understanding of HD is more comprehensive than ever before, and significant effort is being directed towards therapeutic development.

Given that HD is caused by a single gene mutation, in recent years much effort has been directed towards gene silencing approaches that target the mutant HTT mRNA and protein. In addition, efforts are being directed to deliver gene therapies expressing trophic factors to attempt to slow neurodegeneration in HD. Such approaches hold great promise, provided the therapeutic agents such as trophic factors (e.g. BDNF) or antisense oligonucleotides and siRNAs [25, 28, 29] can be delivered effectively to the areas of the brain in which they are needed. However, since these novel therapies have rarely been used in humans, and never in HD patients, several issues need to be addressed before they can move to the clinic. These include whether or not (i) adequate and appropriate biodistribution of the agent can be achieved in the human brain; (ii) the pharmacokinetic parameters are satisfactory (in particular, whether or not a sufficient dose can be delivered to the target site and be efficacious); and (iii) they can be used safely. This last point is particularly relevant, since gene therapies might be active for long periods, and may not be reversible. In addition, many of these novel gene-based therapies are likely to require the use of invasive delivery methods to the brains of patients and such delivery methods are mostly unproven [26]. Finally, potential side-effects of direct delivery, such as inflammatory reactions (e.g. encephalitis), neoplasms (tumours), and unknown changes in neuronal function or metabolism, need to be determined for both short- and long-term exposure. For some therapies, the most efficient method of delivery is undetermined, and improving distribution in brain tissues will need further exploration.

SMALL ANIMAL (RODENT) MODELS OF HD

Numerous transgenic mouse and rat and knock-in mouse models of HD are available for preclinical research (refs [30–42] in Table 1). Genetic engineering technology is well advanced in rodents, and this has allowed the creation of transgenic (carrying gene fragments or the full-length gene), as well as knock-in mouse models of HD carrying varying sizes of the CAG repeat expansion (e.g. allelic series) inserted into the mouse *Htt* gene. Mice in particular are cheap and sustainable laboratory animals, and our understanding of HD has greatly benefited from the accessibility of mouse models. Investigators studying HD are fortunate in that they are able to choose to use the model that is best suited to address their biological questions. The use of mouse models has been very fruitful, and genetically engineered rodent models have provided good insight into the pathology of HD, including an estimate of the extent of the repertoire of changes in gene expression [43], the discovery of neuronal intranuclear inclusions [44], and other aspects of HD such as cognitive dysfunction, and the sleep and circadian disorders that were previously not well recognized as being part of the repertoir of HD symptoms [17, 45]. There are several excellent reviews of the rodent models of HD (see Table 1 and ref [46]) and we do not intend to review these models in detail in this article.

LIMITATIONS OF SMALL ANIMAL MODELS OF HUNTINGTON'S DISEASE

There should be little debate about the importance of rodent models of HD in preclinical research and development. Nevertheless, there are three issues associated with the use of small animals that cannot be addressed by further technological developments.

The first is that mice are short-lived. This necessarily restricts the study of neurological disease in mouse models to a short time period. However, most human neurodegenerative diseases take many years to appear,

Table 1
Comparison of different species as experimental Huntington's disease models

Parameter	Species			
	Rodents	Nonhuman primates	Sheep	Minipigs
Existing transgenic HD models	Mice (partial list) Fragment of mutant *HTT*: R6/2 (exon 1) [30] N171-82Q [31] LV-N171-82Q [32] Full length *HTT*: YAC128 [33] BAC HD [34] Rats Tg51 [35] BAC [36]	Exon 1 *HTT* [63] LV-N171 *HTT* [62]	Full length *HTT*; OVT73 [85]	N208 *HTT* [98] N548 *HTT* Full length *HTT* (Baxa et al.; this issue)
Existing knock-in HD models	Mice (partial list) Q18, 48, 78, 92, 111 [37] HdhQ50, 100, 150, 200, 250, 300 + [38, 41, 42]; Detloff; unpubl. CAG140 [39] zQ175 [40]			Exemplar Genetics (under construction; personal communication)
Gestation period	20 d (mouse); 21 d (rat)	167 d (rhesus)	145 d (Merino)	114-115 d (Tibet; Libechov)
Birth cohort sizes	5–10 (mouse and rat)	1 (rhesus)	1-2 (Merino)	~5 (Tibet; Libechov)
Husbandry	Caged; isolation or group	Caged; colony or isolation (typical)	Natural; outside in paddocks Pens; isolation or groups	Pens; isolation or groups
Lifespan typical	2 years	15 years	15 years	9–14 years
Lifespan possible	3 years	~25 years	~20 years	
Brain size	0.5 g (mouse); 2 g (rat)	~90 g (rhesus macaque)	~130–140 g (Merino)	~90 g (Tibet, Libechov)
Cortex anatomy	Lissencephalic	Gyrencephalic	Gyrencephalic	Gyrencephalic
Cost –purchase of normal adult animal	$20–$30 mouse depending on strain/age $13–50 for outbred, $25–80 for inbred	$5,000 –$10,000 each (USA) Up to 20,000 each (UK)	$200–$600 (USA) $75–250 (UK) (depends on season; sex, origin, transport, health evaluations)	$200–$600 (depending on origin, transport costs, health evaluations)
Cost- maintenance per week per unit	$15–30/week/ cage	$7–$15/day (depending on size, housing, health status) UK $400 per week	$7–$15/day (depending on size, housing, health status)	$7–$15/day (depending on size, housing, health status)
Behavioral testing protocols (Cognitive)	Well established especially in rats Issues of translatability	Established; limited for HD	Not well established	Not well established
In vivo electrophysiology	Routine but *in vivo* is limited due to brain size	Routine	Not routine but technically feasible	Problematic: large sinuses
In vivo imaging	Poor resolution due to brain size	Good resolution	Good resolution	Good resolution
Locomotion	Quadruped	Biped	Quadruped	Quadruped
Safety Issues	Minimal	Significant	Minimal	Minimal
Priority as ethical target	Low	Very high (targeted)*	Low	Medium (targeted)*

*Pigs have been declared as the next focused target for animal rights activists, after non-human primates and dogs.

with HD typically taking 40 or so years to manifest. Time from post-manifest to the end of illness may be up to 25 years. Thus, even if mice become symptomatic, their short lifespan excludes the possibility of extended study. It is not clear if 'clock' time or percentage of normal life span is the critical variable. Irrespective, large animal models would provide the potential for studying HD over a clock time that is closer to the time over which the human condition develops (i.e. 5–10 years or longer).

The second is that rodents in general, and mice in particular, have small brains (Fig. 1). This has an impact on both research application and therapeutic development. For example, advanced measurement techniques such as *in vivo* imaging and *in vivo* electrophysiology cannot be applied optimally in mice. Some of the most important recent advances in neurology have been the development of techniques for examining brain function *in vivo* (magnetic resonance imaging (MRI) and positron emission tomography (PET) scanning), but the resolution of many scanners is an area barely smaller than the mouse striatum. Similarly, with recording techniques such as electroencephalography (EEG), their small brain size means that only 1-2 recording electrodes are typically used on a mouse, compared to 16–22 on a typical human EEG recording (Table 1). Most important for therapeutic development is that delivery of any treatment that is effective in a mouse will need to be 'scaled-up' for humans, particularly for those that need to be delivered directly into the brain, such as trophic factor infusion, transplant therapies, and gene therapies. But it is not clear if this scaling up will need to be done arithmetically, or if larger brains will need relatively less (or more) drug delivered.

The third issue is that rodent brains lack some of the major neuroanatomical characteristics of the human brain. Of particular relevance to HD, in which pathology probably starts in basal ganglia and/or cortex, is that there are major differences in the anatomy of basal ganglia in human and mouse. For example, mice do not have separate caudate and putamen, nor do they have neuromelanin in the substantia nigra and the functional organisation of their basal ganglia is different from that of primates. Furthermore, mice have lisencephalic (smooth) cortices (Fig. 1). The large volume of the human cortex is made even more so by its gyrencephalic (convoluted) anatomy. The human cerebral cortex has a surface area of 2500 cm^2 that far exceeds that of a rat that has a surface area of is 6 cm^2. (For more details of neuroanatomical comparisons, see ref [110]). This evolutionary advantage of humans is going to pose a particular challenge if therapies need to be delivered to the cortex. The lack of the gyrencephalic cortex may be a particularly important limitation of the use of rodent models, if therapies need to be targeted to this region.

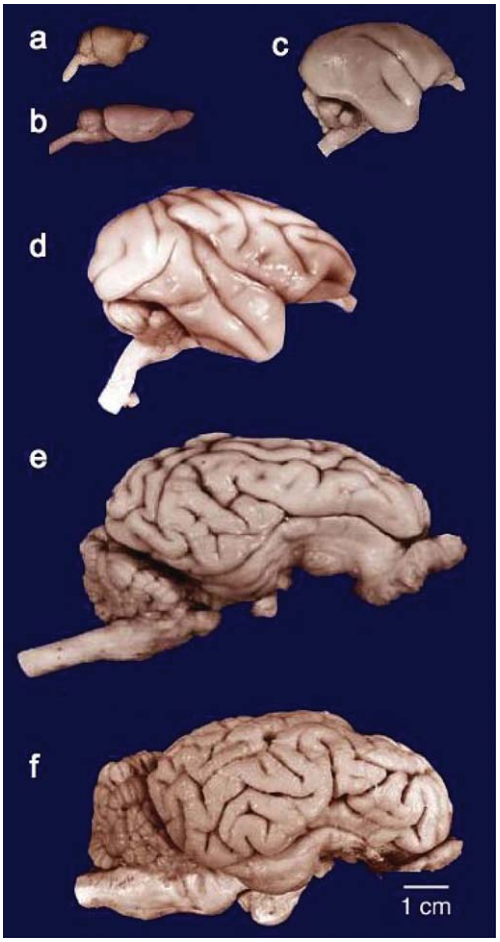

Fig. 1. Comparative brain sizes of species that could be used for modeling neurodegenerative brain disorders. Species illustrated with lissencephalic cortices include house mouse (*Mus musculus*; a), brown rat (*Rattus norvegicus;* b) and common marmoset (*Callithrix jacchus;* c). Those with gyrencephalic cortices include Rhesus monkey (*Macaca mulatta*; d), pig *Sus scrofula domesticus;* e) and sheep (*Ovis aries domestica*; f). The images came from the University of Wisconsin and Michigan State Comparative Mammalian Brain Collections.

THE 'VALUE ADDED' BY THE AVAILABILITY OF LARGE ANIMAL MODELS OF HUNTINGTON'S DISEASE

It has been argued that information gained from using small animals as models is 'good enough' to

allow us to go straight to the clinic. Conversely, it has been argued that animal models *per se* are irrelevant to the human condition. There is also the argument that there are plenty of humans who could be recruited for preclinical studies. But there are several reasons why we think animal models are still needed. For example, the link between gene mutation, brain pathology and symptoms in HD is unknown. While MRI, functional MRI and computer tomography (CT) scans give useful readouts of disease-related changes in structure and function [47–50], they do not inform us about cellular pathology. Brain biopsy in humans is not feasible for practical or ethical reasons, and even if it were, with a disease such as HD that differentially affects areas of the brain, results of a biopsy from one area would not necessarily be representative of what is happening in another. *Post mortem* analysis of tissue is important, but represents a single snapshot in time and is typically at end-stage disease. Studies of end-stage tissue necessarily represent a compendium of events that comprise the toxicity of the mutant gene and its product, as well as a myriad of compensatory sequelae. Understanding the relationship between brain physiology and symptoms in HD would be a key advance that would clarify our understanding of the relationship between HD pathogenesis and its symptomatic progression. It would allow us to identify physiological consequences of the earliest brain changes manifest in neurological disease. It is particularly difficult at present to study the mechanisms underlying conversion from pre-manifest to manifest stages, or even to define those stages unambiguously. Large animals that model pre-manifest (prodromal) HD over a protracted time course would therefore represent a valuable resource with which to study this process systematically. Of course, with the advantages of a relevant timescale come caveats that need be considered carefully. Critically, the cycle time for studies using large animals will be greatly extended compared to such work done in rodents. In addition, feasibility for the average investigator to use large HD animal models will be limited, further restricting the extent of investigation. (See further discussion below).

As well as the difficulties that come with direct studies of human brain, human research participants are a valuable resource that should not be squandered. Although large cohorts of HD patients and premanifest gene carriers are potentially available for clinical trials [51, 52], there are serious ethical and legal constraints on doing experiments with humans, however well informed they might be. And there will be new ethical issues to tackle once multiple potential therapies come on line. For example, once patients are entered for a clinical trial, they cannot be entered for a second trial unless they withdraw from the first. Of particular importance, especially for novel gene therapy or direct protein therapeutic strategies currently being developed, is the need to investigate adequate and appropriate biodistribution, efficacy and safety of these agents prior to advancing into humans. Preclinical studies in rodent models are a clear starting point for gene therapy investigations for HD. However, since all the data should be more directly translatable to the large human brain, moving to larger brained animal models after promising results are obtained in rodents, is a logical (and possibly necessary) step for optimizing delivery and biodistribution, validating on-target mechanism of action, and assessing safety profiles (Fig. 2). Some studies can be done in normal large animals but given that key outcome measures required for preclinical development are likely to be affected by the expression of mutant HTT in the brain, it will be important to at least attempt to advance these studies in large animal HD models.

NON-HUMAN PRIMATE MODELS OF HUNTINGTON'S DISEASE

The ideal animal model for studying human neurodegenerative diseases would be a large non-human primate. Great apes such as chimpanzee, orangutan and bonobo are humanoid and have an estimated 98% of their genome identical to humans and large complex brains that are anatomically very similar to ours. However, the great apes cannot be used for research in the European Union, and there is pressure for a similar ban to be introduced in the USA (see for example, ref [53]). In 2008, The Great Ape Protection Act was introduced to Congress, proposing an end to invasive research and testing of great apes confined within US laboratories. This is already underway, with new research being suspended, and ongoing research being phased out.

The next best theoretical species would be non-human primates such as monkeys, since although their brains are much smaller than those of apes; their genomes and brains (Fig. 1) are similar to humans. A number of different species of monkey have been used for safety studies for neurodegenerative indications (for examples, see references [54–56]) as well as for preclinical studies aimed at cognition (for examples, see references [57–59]). Some neurochemical lesion monkey models of HD have been developed [60, 61],

although they are not commonly used. This is because while they are useful for modeling certain aspects of the motoric impairments and striatal cell loss that is seen in HD, the fact that (i) they are not progressive, (ii) damage is limited to the area(s) directly related to the lesion, and (iii) they do not express the mutant gene, makes them insufficient for modeling a broad range of symptoms and HD pathophysiology.

LENTIVIRUS NON-HUMAN PRIMATE HUNTINGTON'S DISEASE MODELS

Palfi et al. [62] were the first to report on a genetic non-human primate model for HD. This group used a lentivirus encoding a fragment of human mutant *HTT* (*HTT*171-82Q) that was injected directly into the dorsolateral sensorimotor striatum of rhesus macaques. The virus was injected unilaterally, and treated animals that were tested with apomorphine (a dopamine agonist) showed signs of chorea, dystonia and ipsilateral turning. Histological examination of the infected striatum showed evidence of neuritic and nuclear HTT aggregates, reactive gliosis and neuronal cell loss. Bilateral injections of the *HTT*171-82Q virus into dorsolateral striatum resulted in dyskinesia that appeared slowly and then persisted for 30 weeks.

The lentivirus approach has clear disadvantages as well as advantages. The main limitation is that, as with excitotoxic lesions, the restricted expression of mutant HTT in the brain limits the recapitulation of the spectrum of HD pathophysiology. An advantage of the lentivirus model is that since lentiviral injection does not require the generation of germline transgenic animals, the timelines for study are accelerated. Furthermore, since different variations of the *HTT* gene and mutation can be generated, lentivirus-treated animals could be used to investigate the impact on resulting pathology and behavior of different sized fragments of mutant HTT and/or CAG repeat length. Although these models may have only limited utility because of local/restricted mutant HTT expression, the effects of mutant HTT on local neuronal environments can be analyzed. An example of this would be using lentivirus-infected striatum to study pharmacodynamic effects of the local infusion of a gene therapy aimed at reducing mutant HTT expression. Injections of the lentiviruses could also be used to study the impact of mutant HTT expression in different brain regions and circuits. Finally, the described work has already opened the door to investigators pursuing germline transgenic models of HD.

GERMLINE TRANSGENIC NON-HUMAN PRIMATE HUNTINGTON'S DISEASE MODELS

Yang et al. [63, 64] used lentiviral-mediated transgenesis to microinject a human exon 1 *HTT* gene fragment with 84Q and a lentivirus expressing GFP into the perivitelline space of rhesus macaque oocytes to generate transgenic animals. Expression of the mutant HTT was driven by the strong constitutive human polyubiquitin C promoter. Five live newborn founder animals were delivered at term. In these five animals, there was a range in CAG repeat size in the integrated transgenes (from 27 to) indicating that either variants of transgene DNA were present in the DNA preparations used for microinjection, or there was instability of the CAG repeat size during virus production or transgene integration in the embryos. Two of the transgenic animals died perinatally. These animals had multiple transgene copies inserted with CAG repeat sizes of 88 and 27/65. A third animal died after 1 month and showed signs of dystonia and chorea. This animal also had multiple copies of the transgene inserted (with a CAG repeat size of 84 in each of its copies). A fourth transgenic founder carrying a single copy of the transgene with 83 CAG repeats was reported as normal but died aged 11 months after the article was published (personal communication, A Chan). Histological analyses of brain sections from two of the transgenic animals that died very early showed evidence of widespread mutant HTT inclusions present throughout the brain in nuclei and in neuropil. These inclusions were morphologically identical to those seen in R6/2 mice [44, 65].

Further work by this group was done using a microinjected transgene with the ubiquitin promoter driving an exon 1 human mutant HTT gene with 147Q [66]. These transgenic monkeys however did not survive to term, indicating that the higher Q length in these monkeys was very toxic. A single transgenic founder from the original studies survives at the Yerkes Primate Center today [67]. This animal expresses only a single copy of the transgene carrying 29 CAG repeats. Stems cells [68] and induced pluripotent stem (iPS) cells [69] have been successfully derived from the HD transgenic monkeys providing new valuable cell based models. The Emory group continues its efforts to characterize the living CAG29 transgenic monkey, and have also extended their work to generate additional HD non-human primate transgenic models that have not yet been reported in the literature.

LIMITATIONS OF THE USE OF HUNTINGTON'S DISEASE MONKEY MODELS

HD transgenic monkeys may be particularly useful for following early disease development and pathology, and in the application of sensitive imaging techniques (such as MRI and PET) to provide non-invasive measures of disease. Additionally, HD non-human primates could be a valuable resource for studying the effects of mutant HTT on cognition, if such data are translatable to the human condition. Testing of therapeutic candidates using each of these measures would provide valuable preclinical data in terms of both efficacy and safety, helping to inform human clinical trials. However, there are a number of major hurdles to be overcome before non-human primates will be acceptable as a large animal model for use in HD preclinical research (see Table 1). First, although the number of animals that need to be used at any one time would be very small, availability of HD animals will be at a premium. Typically, for behavioural testing in normal animals group sizes of 6–12 are used; for pharmacological safety trials, groups of 12–20 animals are used. Normal monkeys can be obtained relatively easily for experiments, but HD non-human primates would need not only to be developed, but also to be bred in sufficient numbers and maintained until old enough to use in preclinical trials. Also, even normal monkeys are costly to purchase and house. Currently in the USA, typical purchase costs for a rhesus macaque are $5000–$10,000, and costs, including for animal maintenance, are higher in the UK (Table 1). Importantly, there are also indirect costs –not least the ethical issues that surround the use of non-human primates for research. Indeed, many universities are reducing their use of non-human primates in response to public antagonism.

In addition to the high costs, the practicalities of housing monkeys with a slowly progressing neurodegenerative disease will be challenging. If the animals manifest HD symptoms that recapitulate the human disorder accurately, we would expect not only motor symptoms, but also psychiatric disorder and cognitive decline. The management of these symptoms in the context of experimental testing presents both a practical and an ethical challenge that will need to be addressed. It is extremely difficult to manage ill animals within a monkey colony. The normal practice is to isolate them so that their medical needs can be managed effectively and safely. For short-term illnesses, this is practicable. But long-term isolation of non-human primates causes stress that would add confounds that are difficult to control. Once animals start to show symptoms, decisions about their optimal care may have serious impact on the nature and duration of experiments. Questions that need addressing include (i) how will isolation stress or experimentation affect the disease progression? (ii) How could this be controlled? (iii) Would there be a need either to treat symptoms (such as depression, psychosis) or to tranquillize monkeys for transport, and if so, how would these affect testing? (iv) At what age should experiments be started? and (v) How large do group sizes need to be for well-powered studies? Finally, human HD patients typically need round the clock nursing care in the last 5–10 years of their lives. This would not be possible with HD monkeys, so it is likely that only pre-manifest and early-mid stage disease could be studied. Diseases such as HD that include psychiatric disturbances could also increase the risk of injury to either the investigators or the animals. Lastly, although transgenic technologies have been established in non-human primates, the technology for making knock-in or gene-targeting modifications in non-human primates has not advanced to the stage where viable animals have been born [70]. The development of HD monkeys is likely to be undertaken by only a small number of investigators, and access to such animals will be limited, and probably beyond the reach of the average investigator.

FARM ANIMAL MODELS OF HUNTINGTON'S DISEASE

The most commonly used second species for therapeutic testing in Europe is the dog. But to our knowledge, there is no HD research group currently using dogs as experimental animals.

Large brained domesticated farm animals represent interesting possibilities for the development of new models of HD. The farming industry already has developed well-defined systems for breeding, rearing and transporting such animals. However, there are relatively few domesticated animals that might be useful for HD research. Horses, donkeys and cattle are theoretically possible, but they are very large animals, and uniparous with relatively long gestation periods. Thus, the costs of breeding and rearing, as well as the risks and issues of manageability associated with their size, would outweigh any possible advantages. For the remainder of this review therefore we will focus on the potential of sheep and pigs as large models of HD. (Sheep and goats share similar physiological and

behavioral characteristics, so for simplicity, here we will discuss only sheep).

SHEEP AS MODELS OF HUNTINGTON'S DISEASE

Sheep fulfill a number of simple practical requirements for a species in which to model HD. They are domesticated, but are relatively outbred. They are docile and pose little risk to the investigator. They normally live in flocks outdoors, so they do not require specialized housing. They are therefore relatively easy to care for and are cheap to maintain. Because sheep can be kept in their natural environment outdoors, there are no issues of impoverishment of environment associated with use of conventional laboratory animals. Furthermore, they are economical to use as research subjects. Sheep do not continue growing throughout their lives. Once they reach maturity, no modifications to their housing and equipment are required. Depending on the breed, their body weight is comparable to an adult human. For example, in a large breed such as the South Australian merino, ewes reach a mature weight of 50–70 kg by 2 years of age. This makes it feasible to use them for systematic therapeutic testing.

Sheep are long-lived compared to rodents. A mouse is 'old' by 2–2.5 years of age. The natural lifespan of a sheep is typically 12–15 years, although they are capable of living for >21 years. This longer lifespan makes them suitable for studying much later stages of progressive neurological diseases than is possible in rodents.

Physiologically, sheep make practical models. Although they are ruminants, their other body systems are very similar to humans, and they have been widely used as large animal models of reproductive biology, respiratory and cardiovascular physiology, joint surgery and as host organisms for viruses. Their brain biology is less well studied, with the exception of hypothalamic physiology, which in sheep is particularly well understood since their reproductive system is so similar to humans (see for example, ref [71]). Nevertheless, although many aspects of sheep brain physiology have not been extensively studied, some seminal discoveries have been made using sheep, particularly in the field of facial recognition [72, 73]. Furthermore, new directions in the use of sheep, for example as models of cognitive function [74] are emerging.

Their physical size, although a cost disadvantage, is an advantage where experimental designs are concerned. Sheep are strong and can carry a backpack with transmitting devices. This means they can be used for ambulatory telemetry experiments using equipment designed for humans. This cannot be done easily in mice because they are too small, and is difficult to do non-invasively in monkeys, because they are dexterous enough to remove the data collection modules.

SHEEP BRAIN SIZE AND ANATOMY

One of the best reasons for considering sheep as a large animal model of human brain function is their large brain. An adult human brain weighs 1300–1400 g. Non-human primate brains are also large (an adult orangutan brain weighs 360 g, a rhesus macaque brain weighs 90–97 g). A mouse brain weighs 1gram. By comparison, an adult sheep brain weighs ~130–140 g (refer to Fig. 1 for comparisons). The large brain of sheep allows the use of *in vivo* measuring techniques of brain structure such as MRI [75–78]. The larger brain size also means that strategies devised for drug delivery directly into the brain in the sheep may translate well to humans. For the same reasons, sheep represent a good species as a 'follow-on' to early preclinical studies done in mice or rats, particularly for testing cell replacement approaches, or the delivery of gene therapy agents (Table 1 and Fig. 2).

Sheep brains are anatomically more similar to human brains than are the brains of mice. Sheep not only have gyrencephalic cerebral cortices but also more human-like subcortical structures than mice (Fig. 1). The sheep brain has a clearly identifiable separate caudate nucleus and putamen, and sheep thalamic nuclei are also more comparable to the human than those of the mouse. Furthermore, the GPi and substantia nigra in sheep are similar to those in non-human primates, both in size and functional organisation.

SHEEP BEHAVIOUR

Although sheep have a reputation for being stupid, this is probably undeserved, and based more on casual observation of flock behavior than it is on systematic testing. The fact that sheep have rarely been used for cognitive studies is more likely to be on the basis of practicality than ability. In fact, sheep exhibit all the behaviors that make rodents useful subjects for behavioral testing. They have good memories and are capable of learning and remembering new tasks [74, 80]. Sheep can perform some cognitive tasks that are extremely difficult to test in mice and rats, such as the intradimensional/extradimensional set shifting task that is considered to be a measure of executive function [74].

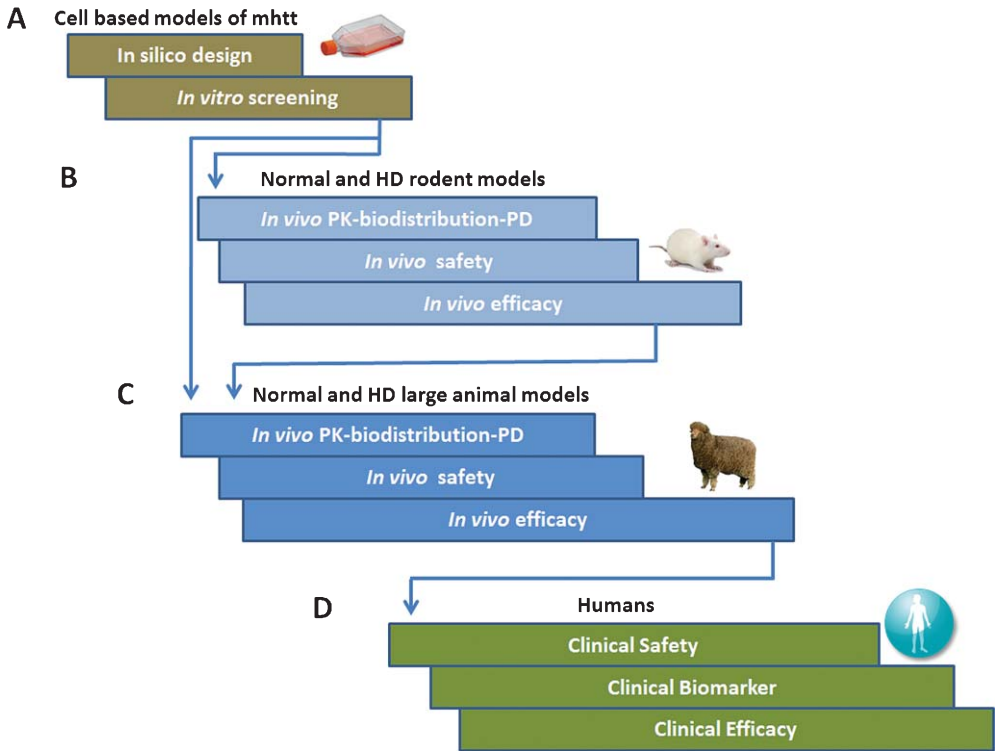

Fig. 2. A modified scheme for preclinical development of a gene based therapeutic for Huntington's disease. *In silico* design is typically followed by testing of candidate reagents in cell based models (a) for selecting those that are engaging the target of interest (i.e. the mutant *HTT* gene). Currently, gene therapies are tested for pharmacokinetic exposure, biodistribution in the CNS, specific activity (pharmacodynamics measures) and efficacy in normal and HD small animal models (b). A proposed inclusion (or eventual replacement for (b)) is the testing of gene therapy agents for PK, biodistribution, safety and efficacy using large animal models of HD (c) prior to proceeding for clinical testing in patients (d).

They also exhibit excellent facial (for references, see [72]) and olfactory recognition [79, 80].

NEUROLOGICAL DISORDERS IN SHEEP

Sheep develop progressive neurological diseases. Where these diseases are similar to those transmitted to humans (for example, new variant CJD), the progression of the disease in sheep is similar to that seen in human disease [81, 82]. Interestingly, it is emerging that there are a number of natural mutations found in sheep that cause neurological diseases with pathology similar to that seen in humans with similar mutations. For example, there are a number of sheep models of lysosomal storage diseases, such as Batten's disease [83] and Gaucher's disease [84]. Sheep also show human-like neurological symptoms in response to poisoning with neuroactive toxins. This raises the expectation that the HD mutation should cause a sheep form of HD with progression similar to that in humans.

HUNTINGTON'S DISEASE TRANSGENIC SHEEP

A number of lines of transgenic HD sheep have been developed in New Zealand and Australia [85]. These lines were made by pronuclear injection of a full length cDNA of human *HTT* carrying an uninterrupted 73 CAG repeat and driven by a human *HTT* gene promoter. From 127 Merino lambs born resulting from embryo transfers, six lambs (4 rams and 2 ewes) were identified as being transgenic for the human *HTT* transgene. The transgene integrated at a single genomic locus in 5 of the 6 transgenic founders but in multiple copies ranging from 2 to 14. Whole genome sequencing and targeted capture of breakpoints with capillary sequencing has been performed on the HD transgenic sheep DNA to identify the chromosomal site of integration and *in situ HTT* transgene sequence [86]. The sequencing data showed that all of the founder HD sheep had evidence of transgene DNA shattering, leading to complex rearrangements of the DNA in the sheep chromosomes. However, intact full-length *HTT* trans-

gene copies were evident in some of the transgenic founders including line Go/5 that has been the most well studied line. Based on skin fibroblast expression levels of mutant HTT mRNA and protein, founder Go/5 (OVT73, formerly known as the Kiwi line) was prioritized for breeding to F1 and further analyses. Its progeny express mutant human HTT mRNA and protein ubiquitously in the brain [87] and develop HTT aggregates and inclusions that are evident in various brain regions by 18 months in age (Patassini et al., personal communication). Jacobsen et al. [85] reported the dysregulation of DARPP-32 expression in the caudate and putamen of a single 7-month-old OvHD73 transgenic lamb, but this needs to be confirmed with larger number of animals. Although none of the founders showed any significant early pathology or overt phenotype, this was not unexpected, given that the mutation in the transgene is of a length that in humans would not be expected to manifest disease until late childhood. The oldest HD sheep (G1), currently 5 years old, are the subject of a comprehensive collaborative phenotyping initiative being conducted by Snell and colleagues (University of Auckland, NZ), Morton and colleagues (University of Cambridge, UK) and Bawden and colleagues (South Australia Research and Development Institute, Australia). The HD sheep as yet show no overt symptoms, however in-depth investigations are currently in progress. Only time will tell if the disease in these sheep progresses to a stage that they recapitulate phenotypic symptoms similar to human HD. Nevertheless, even without symptoms these HD sheep are a valuable resource. For example, because they carry a human transgene that is expressed at both an mRNA and a protein level, they could already be used for testing of gene therapy based reagents directed against human HTT (Fig. 2). In the context of the large animal brain, this would be useful for studying biodistribution and the relationship of pharmacokinetics (dose/efficacy) to the extent of HTT reduction in brain, as well as long-term safety (Fig. 2).

DIFFICULTIES RELATING TO THE USE OF THE HUNTINGTON'S DISEASE TRANSGENIC SHEEP

There are a significant number of hurdles to overcome before sheep can be used routinely as large animal models of HD. First, the sheep genome is not fully annotated. This creates difficulties in currently applying the full power of genomics technologies such as Affymetrix profiling and RNAseq for the analyses of genes and pathways that may be dysfunctional in any sheep model. Progress in this regard is being made in sequencing the sheep genome and establishing a transcriptome (see HTTp://www.animalgenome.org/sheep and ref [88]). Second, many behaviors that are easily and routinely assayed in rodents (locomotor function, cognitive function, anxiety) have never been quantified in sheep in a laboratory-like setting. Standard protocols for behavioral testing (such as those currently available for rodents) need to be established if they are to be used for cross-study comparisons. HD-relevant behaviors that would be useful to measure in HD sheep include cognitive, social and psychiatric function, but these behaviors are poorly characterized in sheep. Third, sheep are uniparous in breeding, and produce only a small number of lambs per year (1–3 per breeding ewe). Therefore a large number of breeding ewes is required to scale the breeding adequately for generation of the large numbers of animals that might be required for behavioral studies. Fourth, sheep are quadrupeds. This means that any motor symptoms are necessarily going to be different from that in bipedal humans; this is particularly important when considering the chorea that is characteristic of adult HD. Nevertheless, chorea is not the only motor symptom of HD, and many of the other abnormalities in motor function seen in HD (rigidity, dysphagia, bradykinesia) have been identified in large quadrupeds [89]. Finally, sheep are ruminants. Rumination is a distinct disadvantage both for oral drug delivery and the study of the digestive system. But for translational drug discovery research, the great strength of a large animal model for HD lies with investigation of therapies aimed at direct administration to the large brain as opposed to investigation of small molecules given by oral dosing. Therefore sheep rumination is not likely to present an insurmountable hurdle to testing drug delivery.

PIGS AS MODELS OF HUNTINGTON'S DISEASE

As with sheep, there are considerable advantages for using the pig as a laboratory species, especially with regards to the similarities between human and porcine brain structure and function. Pigs have brains similar in size and structure to sheep (Fig. 1). They have relatively long lifespans (12–15 years), and so they should be useful for studying early changes in neurodegenerative diseases, with potentially long prodromal periods (Table 1). Physiologically, pigs make a 'close to ideal' model for humans. All of their body systems are very

similar to humans, including their digestive systems. As such, they have been widely used as large animal models of reproductive biology, respiratory and cardiovascular physiology (see ref [90] for review). Pigs are domesticated, but remain relatively outbred. In the wild, female pigs live in small groups; males are solitary and intact males cannot be housed together. But castrated males do not pose this problem, and pigs are routinely raised in large numbers for the food industry, so husbandry methods are well established. Pigs raised for food are rarely kept in natural groups outdoors, so there are issues of impoverishment of environment associated with 'laboratory' animals. But they are economical and practical to use as research subjects. There has been substantial progress in sequencing the porcine genome [91–94] making application of gene profiling studies such as RNAseq useful for mapping gene and network dysregulation in an HD minipig. Furthermore, genetic manipulation techniques that allow for transgenesis and homologous recombination are well advanced in minipig strains [95–97] such that mutant HTT could be engineered in minipig in multiple ways.

HUNTINGTON'S DISEASE MINIPIGS

Yang et al. [98] reported on the successful generation of transgenic HD minipigs using somatic cell nuclear transfer technology. Two transgenes were constructed, a human *HTT* N208-105Q-ECFP and an N208-160Q-ECFP each where expression was driven by the strong constitutive chicken beta-actin promoter with a cytomegalovirus enhancer. Reconstruction of porcine embryos after injection of the N208-160Q-ECFP transgene did not result in any live births, suggesting that this *HTT* fragment harboring a very high CAG repeat length resulted in embryo toxicity. In contrast, there were five live births carrying the N208-105Q-ECFP transgene. Although transgenic piglets appeared normal at birth, three of the piglets died within 3 days, and a fourth lived for only 25 days. The fifth founder was still viable at time of publication. This animal showed the lowest expression of the mutant HTT fragment of all the transgenic offspring, which suggests that high levels of mutant HTT expression contributed to an early death phenotype in the other piglets. Histological analyses of brain sections from the piglets that died early showed evidence of neuronal EM48 antibody immunoreactivity, verifying presence of mutant HTT. Neuronal cells positively stained for mutant HTT also showed evidence of fragmented DNA, a sign for cell apoptosis.

A new HD minipig model has been developed by Motlik and colleagues at the Institute of Animal Physiology and Genetics in the Czech Republic (Baxa et al., 2013, this issue). In this line, lentiviral based transgenes were constructed carrying a human minimal *HTT* gene promoter driving either a 548 amino acid or full length (3144 amino acid) human *HTT* cDNA containing 145 repeats of a mixed CAGCAA sequence. Founders for each the 548 amino acid fragment and the full length *HTT* transgenes were generated. The 548 amino acid founder has been bred successfully out to the G3 generation. The oldest of these HD minipigs is approximately 3 years at the time of writing, and do not show overt disease symptoms. Thus, as with the HD sheep, they have great promise as useful HD large animals, potentially modeling the slow and progressive nature of HD, although this promise has not yet been fulfilled. Evidence of mutant HTT expression in brain has been shown along with some specific biochemical phenotypes including reduced DARPP-32 expression in the striatum of 16 month old HD minipigs as well as reduced sperm cell motility and oocyte penetration, an indication that the mutant HTT is having effects at early ages. These HD minipigs are the subject of a comprehensive phenotyping initiative being conducted by Reilmann and colleagues (University of Munster) where the plan is to use outcome measures informed by human HD observational studies (TRACK-HD) in premanifest patients [99, 100].

RELATIVE MERITS OF SHEEP AND PIGS AS MODELS OF HD

With both pigs and sheep, the big advantage for long-term studies is the wide experience of traditional agriculture that increases the feasibility of long-term studies. In some respects, in particular, pigs have clear advantages over sheep. While their gestation times are similar (113–115 days), pigs are multiparous and produce large litters (of up to 10 piglets). Breeding sows can have 2 litters a year. This is an obvious advantage where generation of large numbers of animals is desired. Pigs are omnivores, and have a digestive system very similar to humans [90]. This contrasts favourably with sheep, which are ruminants and obligate herbivores.

But there are also disadvantages of pigs compared with sheep. Adult males do not live in social groups and uncastrated boars need to be housed individually. Female pigs live in groups, but among animals in the same litter a strong social hierarchy is established

within a few days after birth and persists throughout their lifetimes, which is likely to be an important factor in social behavioural testing. Both pigs and sheep are docile and pose relatively little risk to the investigator. When there is a risk however, this is likely to be greater for pigs than for sheep, since the natural instinct of a sheep in a threatening situation is to flee, whereas that of a pig is to challenge. Body size may also turn out to be an issue. Pigs continue to grow throughout their lives, and most farm pigs grow too large to be practical for experimental use. There are several miniature and micro-pig breeds that have become successful laboratory models [90]. Micropigs will not be considered further as models for HD, since any advantage they have in small body size is lost because their brains are correspondingly smaller as well. Minipigs are likely to be the species of choice, because they are already relatively well characterised. It should also be noted however that 'mini' is a relative term. Minipigs are small compared to landrace pigs that can achieve body sizes of 400–600 pounds by 2 years of age. But the Tibetan and Libechov minipigs that have been used to make the HD models as described above are not small animals –indeed, a minipig can grow substantially, and could weigh up to 250 pounds or more as an adult, depending on its genetic background. This large body size, coupled with the associated strength will make conducting behavioural testing with these animals an interesting challenge.

The feasibility of stereotactically-guided delivery of drugs into the brain has been found to be good for both pigs [76] and sheep [77]. Sheep have an advantage over pigs where implanted brain devices are concerned. Pigs have large skull sinuses that preclude long term implants. (Long term EEG studies have not been successfully achieved in pigs for this reason). Sheep have a different skull anatomy that makes long-term recordings from sheep brain feasible. Indeed, we have recorded EEGs from sheep brains for up to a year using skull electrodes (AJM, unpublished data).

Both pigs and sheep are easily anaesthetized and amenable to *in vivo* imaging modalities applied in humans. Imaging has been conducted much more widely in pigs than in sheep. MRI, fMRI and nuclear magnetic resonance spectroscopy studies have all been done in pigs [101–103] as has PET [104–106]. Nevertheless, both MRI and CT scanning have been conducted successfully in sheep ([77, 107]; and AJM, unpublished data).

Pigs are generally considered to be 'smarter' than sheep, although direct evidence for this is lacking. Indeed, neither species has been used routinely in the laboratory for cognitive testing. This is a hurdle to the use of both species for behavioral studies, since there are no well-established tests for systematically measuring cognitive function in either pigs or sheep. In contrast, cognitive testing is well established in monkeys, an advantage for modeling in non-human primates.

LARGE ANIMAL MODELS OF HD: WHERE ARE WE HEADING?

We recognize that no single animal model can fulfill all of the requirements needed to address the many remaining questions in HD research and development. We also recognize that it is unlikely that the power of genetics, as has been used in lower organism and mouse models of HD to uncover developmental and cell biological consequences of mutant HTT, could be applied as effectively to monkey, sheep or minipig models. Nevertheless, large HD animal models are already proving to be interesting supplements to the arsenal of lower organism and small animal models of HD that have been used to such good effect over the last two decades. For example, the published work reported in the transgenic rhesus macaque represented a first significant leap forward as a proof of concept that a fragment human *HTT* transgene with expanded CAG repeats results in a severe pathophysiology. Histological analyses of the brains from these monkeys showed classical nuclear and neuropil aggregates that are similar to the histopathology seen in rodents. Some of the arguments about the relevance of aggregates in rodents to primate disease should now be laid to rest. The data from transgenic HTT fragment minipigs extended the findings from the transgenic non- human primates showing that high level expression of a fragment of the human gene recapitulates a histological mutant HTT phenotype and results in early behavioral deficits.

Renewed efforts are underway to make models of transgenic non-human primates, minipigs as well as full-length transgenic HD sheep in which disease develops more gradually. These long-lived HD animal models therefore hold great promise for recapitulating the slow and progressive nature of HD and providing an invaluable resource for HD research and drug development. It will be important to include them in preclinical testing designs, especially for gene therapies as data obtained here will be valuable for predicting biodistribution, safety and efficacy in HD patients (Fig. 2).

The work described in this review represents significant steps toward modeling HD in large animals.

However, there is one limitation of all of the large animal HD models described here. They are all transgenic thus each model expresses two copies of their own normal *Htt* genes as well as the extra copies of *HTT* introduced via a transgene. None of these models recapitulates HD at the genetic level, where there is typically one normal allele and one mutant allele. The potential impact of overexpressing total HTT in mouse models is recognized [108, 109], but not well understood. Nevertheless, it is likely to play a role in the progression and manifestation of the disease, and remains a caveat. In an effort to alleviate this problem in part work is underway at Exemplar Genetics (Sioux City, IA) to generate a knock-in model of HD in the minipig.

New large animal models of HD are being developed by multiple groups, and their characterization is progressing apace. Importantly these large animal models are being studied for disease phenotypes using sensitive measures that should be highly translatable to the human condition including MRI, and PET imaging, EEG, electrophysiology, molecular analyses including RNAseq, in addition to tests looking at motor, and cognitive function. With current efforts from groups working on transgenic HD non-human primates at Emory Yerkes Primates Center, USA, the viral-based mHTT expression in non-human primates at the Oregon National Primate Research Center, USA, the transgenic HD sheep at SARDI in Australia and transgenic HD minipigs in the Czech Republic, there is good likelihood that large genetic HD animal models will eventuate, that can be used not only to study pre-manifest and manifest disease, but also to test delivery of gene-targeted therapies in a large brain context.

This review has discussed a new direction for modeling of disease in animals. But alongside the new models comes new challenges. None of the animal models discussed above are ready to be used by the research community. However, if they are to be used optimally once they become available, the HD research community will need to take a new approach to some practical aspects of preclinical research. Currently, most experimental programs depend on an individual researcher's personal interests, and their ability to apply successfully for research funding. The design of all experiments requires careful planning, but experiments that might last 5 or more years require not only careful planning, but also critical evaluation of scientific priorities. Research funding will also need to work to a different model. With grant cycles rarely exceeding 3 years, studies that might take 5–10 years are currently not feasible, and will not be possible with existing models of funding. Finally, given that large animals have requirements for space that are not available in most city-based universities, unless a creative approach is taken to sharing animals, access for the average investigator to large animal models of HD will be limited. Thus the valuable resource, that large animal models of HD should become, will be underused. Long-term collaborations, as well as pooling of resources, will become much more important for experiments using large animal models than they currently are with rodents. Although large animals will necessarily remain the subjects of 'boutique' studies, strenuous efforts should be made to develop a mechanism whereby researchers who do not have the appropriate facilities to set up their own studies can negotiate access to animals for their experiments. Neither the mechanism nor funding for this type of long-term collaborative research currently exists.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

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