

Review

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Genetically engineered pig models of neurological diseases

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Abstract

Genetically modified animal models are commonly used for *in vivo* studies of human diseases. Mice are the most common animal models used in biomedical research, which have provided important insights into disease pathogenesis and are widely used to find treatments for diseases. However, due to the differences in the anatomical structure and physiological function between human and mouse brains, most genetically modified mouse models cannot fully recapitulate the overt and selective neuronal loss seen in age-dependent neurodegeneration diseases. While non-human primates (NHP) are closer to humans and have been used to model human disease, these models are difficult to be utilized at a large scale due to various limitations including their high costs, prolonged breeding time, community concerns for use of NHP, and high ethical standards. As an important animal resource in agriculture, pigs are also used as animal models in biomedical research. The central nervous system of pigs is highly similar to that of humans, making pig models suitable for investigating neurological diseases. The relatively short breeding period, large litter size, and established somatic cell transfer technology are advantages over NHP for using pigs to model human diseases. The recent development of gene editing tools allows



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one to more efficiently generate pig models that can precisely mimic genetic mutations in neurological diseases. In this review, we summarize recent advances in the use of pigs for modeling human neurological diseases, including new approaches for generating genetically modified pig models.

Keywords: Pig models, neurological diseases, gene editing, genetic modification, genome editing tools, disease models

INTRODUCTION

In vivo experiments using laboratory animals are essential for the verification of important findings from *in vitro* studies. In addition, animal models of human diseases are critical in revealing pathological changes and disease pathogenesis, which provide the theoretical basis for the development of treatments and therapeutic strategies. Small animal models such as mice and rats have been widely used in biomedical research, and animal models generated from mice have greatly advanced our understanding of the pathology and mechanisms of diseases. Small animals can partially mimic the symptoms and pathologic phenotypes of human disease, especially in extremely complex neurodegenerative diseases. That may be due to the considerable differences in development, aging, and fine structures between mouse and human brains. For example, the full development time for mouse brains is 21 days while primates' brains need more than 150 days to reach full maturation^[1]. The short lifespan of rodents is another major difference that may cause the different presentation of the neuropathology, since mice can only live for a little over two years, which is much shorter than the human's average of 70 years. Therefore, the rapid development of the brain and the short lifespan of mice may cause neuronal cells to respond less strongly to the production of misfolded toxic proteins than do human neuronal cells. Differences in neural circuits and anatomical and physiological features between rodent and human brains suggest that we should explore other animal models to develop neurodegenerative diseases.

Undoubtedly, non-human primates (NHP) are ideal animal models that can closely mimic human diseases due to the high similarities between NHP and humans in genetics, physiology, development, social behaviors, and cognition. However, it is difficult to create a genetically modified NHP model when compared with small animals due to various factors, including long breeding cycles, lack of effective methods for genetic manipulations, high costs, community concerns, and high ethical standards. As a result, the first transgenic mouse model was generated as early as 1974^[2], but the first genetically modified monkey model did not appear until 2001^[3].

Considering the shortcomings of small animals and non-human primates in modeling human neurological diseases, pigs have some advantages over other species. Pig models have several unique features that make them a promising alternative animal model^[4]. Pigs can produce larger litters and have a shorter maturation and reproduction time with fewer concerns about ethical issues and lower costs than non-human primates^[5,6]. In regards to the similarity of pigs to humans, pigs are also highly close to humans in terms of anatomy, physiology, and metabolism^[5]. As for the brain, the central nervous system of pigs is very similar to that of humans. For example, both human and pig brains have many sulci and gyri. Anatomically, the dorsal striata of the pig and human brains are both split into two distinct structures of the caudate nucleus and putamen, compared with a single structure in the rodent brain. In addition, the hippocampus in the pig brain more structurally resembles the human hippocampus than that in rodents. The timing of myelin formation in pig brains is also similar to that of humans during brain development^[6] [Figure 1]. These similarities make the pig a better animal model for studying neurological diseases. In addition, pigs have the advantages of early sexual maturity (5-8 months), a short reproduction cycle between generations, and a

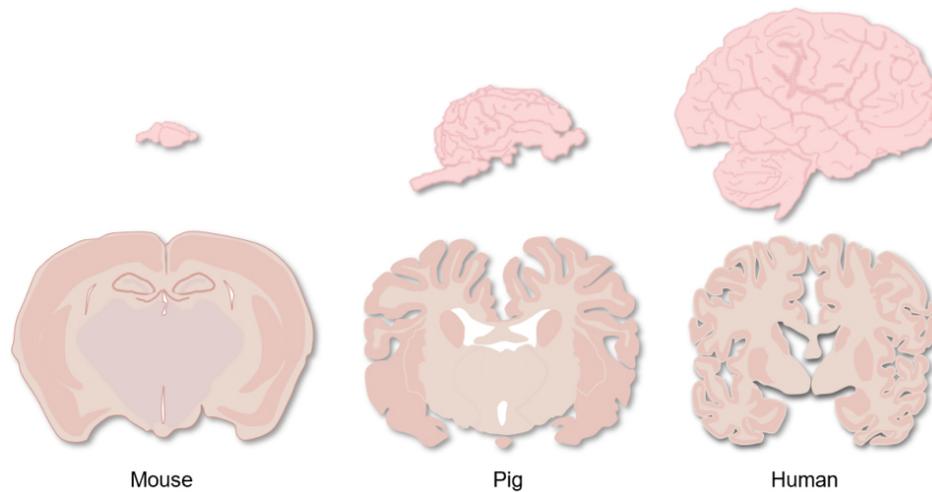


Figure 1. Comparison of brain structures of mouse, pig, and human.

larger litter size (about 10-12 piglets per litter)^[7,8]. Moreover, fully established somatic cell nuclear transfer (SCNT) technology combined with recently developed genome editing technology has made it possible to efficiently generate genetically modified pig models^[9] [Figure 2]. Here, we briefly discuss how to use related techniques to establish genetically modified pig models and review the established pig models for neurological diseases.

METHODS FOR GENERATING GENETICALLY MODIFIED PIG MODELS

For a long period of time, there have been two main methods to establish genetically modified pig models: embryonic microinjection and SCNT. Microinjection is a traditional method for creating transgenic animals and involves injecting DNA material directly into the pronucleus and transferring the early embryo into the surrogate mother to create a transgenic animal, which introduces transgenes randomly into the genome of the resulting offspring^[10]. This method is fairly straightforward, but the efficiency of producing transgenic animals is relatively low, about 10% in mice, 4% in rabbits, and only 2%-3% in pigs^[11,12]. Although several strategies have been used to improve the efficiency of embryonic microinjection, including pronuclei or cytoplasmic injection of DNA or mRNA^[13,14], there are still many difficulties in using this method to generate genetically modified pig models. For example, due to the high lipid content and low transparency in pig oocytes^[15], it is difficult to perform embryonic microinjection. In addition, this method will lead to random integration and poor precision of gene targeting. To improve the accuracy of gene editing, researchers developed a gene targeting strategy using homologous recombination (HR) in embryonic stem (ES) cells, which greatly improves the efficiency of generating gene-targeted animal models^[16,17]. The lack of ES cells in pigs hinders the generation of precise genetically modified pig models. To overcome this difficulty, researchers firstly screen and identify the precisely targeted transgenes in cultured pig cells and then use them for SCNT, making it possible to establish gene-targeted pig models. However, the efficiency of HR in modifying pig somatic cells is very low, and the fatality rate is high due to the intrinsic genetic defects^[18]. Later, an attempt to improve the efficiency of pig gene targeting was made by the application of several important technologies, including the delivery of gene-targeting vectors using recombinant adeno-associated virus (rAAV)^[19,20].

GENOME EDITING TOOLS

Due to low targeting efficiency, for a long time, only a few transgenic pig models had been successfully

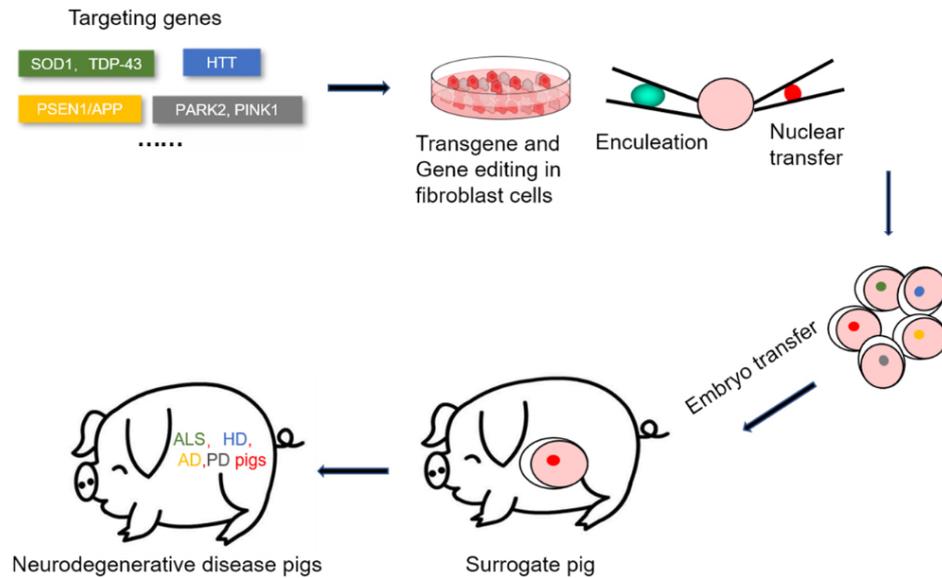


Figure 2. Flow chart of transgenic and gene editing using SCNT to construct neurodegenerative disease pig models. SCNT: Somatic cell nuclear transfer.

established^[21-24]. This situation was greatly improved with the development of new precise gene editing tools, which include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas). ZFNs, composed of DNA-binding domains consisting of tandem zinc finger motifs with nuclease domains from the endonuclease FokI, can induce the targeted DNA double-stranded breaks (DSBs) that lead to DNA damage repair mechanisms^[25,26]. Although ZFNs have been widely applied in many species, including plants, animals, and mammalian cells in culture^[25], they have not been used to create large animal models.

TALENs are an alternative tool for genome engineering^[27-29]. They are also fusion proteins of tandem repeats of a TAL effector protein and the FokI nuclease. TALENs induce the targeted DSBs that activate DNA damage response pathways and lead to gene knockout (KO) or knock-in (KI)^[30]. As compared with ZFNs, TALENs are easier to design and synthesize, and some animal models of disease have been successfully established using TALENs^[31].

Although ZFNs and TALENs have been applied to various species, CRISPR/Cas9 is now the most widely used genome editing tool for generating genetically modified animal models. The CRISPR/Cas9 system confers targeted gene editing by small RNAs that guide the Cas9 nuclease to the target site through base pairing^[32]. When the complex is located at the targeting site of the genome, Cas9 cuts both strands at a precise location. Then, the repair mechanism kicks in to rejoin the damaged genomic DNA by non-homologous end joining (NHEJ) or homology-directed repair (HDR), which may result in mutations to inactivate or alter gene function. Based on this damage-repair mechanism, scientists have optimized the CRISPR/Cas9 system to create many genome editing models for small animals, such as mice^[33], rats^[34], and zebrafishes^[35]. Large animal models such as pigs have also benefited from this technology. Here, we focus on genetically modified pig models of neurological diseases.

Pig models of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult-onset progressive neurodegenerative disease caused by the selective death of motor neurons (MNs). With the occurrence of aging, patients with ALS develop

progressive loss of upper and lower MNs, muscle atrophy, and eventually paralysis, and they usually die within 3-5 years after the onset of symptoms^[36,37]. Currently, the pathophysiological mechanism of ALS remains to be fully understood. Genetic studies have identified more than 30 gene mutations that are highly associated with the etiology of ALS, including copper/zinc superoxide dismutase 1 (*SOD1*) and TAR DNA-binding protein 43 (*TDP-43*). Mutations of these genes affect many cellular and molecular processes, leading to increased oxidative stress, mitochondrial dysfunction, excitatory toxicity, neuroinflammation, protein aggregation, and abnormal RNA metabolism. The neuropathology of ALS is characterized by protein aggregation and accumulation of ubiquitinated protein inclusion bodies in the neuronal cytoplasm. In most ALS patients, *SOD1* and *TDP-43* are the main components of these inclusion bodies, suggesting that *SOD1* and *TDP-43* are causative factors for the occurrence and development of ALS. Therefore, several studies have generated pig models that express mutant *SOD1* or *TDP-43* and showed ALS-like phenotypes.

Chieppa *et al.* produced an ALS pig model using SCNT in combination with transfected somatic cells expressing the G93A mutation of human *SOD1*^[38]. In 2014, Yang *et al.* used similar techniques to generate transgenic pigs that express the same *SOD1* mutation. The transgenic pigs developed age-dependent neuropathology and movement disorders, which recapitulate the features of the early disease symptoms seen in human ALS^[9]. Moreover, transgenic mutant *SOD1* pigs show intranuclear inclusions and an association of *SOD1* with the nuclear protein PCBP1, which were not seen in mouse brains^[9]. In addition to *SOD1*, researchers also established transgenic miniature pigs expressing mutant *TDP-43*. They found that transgenic *TDP-43* was also distributed in the cytoplasm of neuronal cells resembling the pathology seen in human ALS brain tissues^[39], which was not found in many transgenic *TDP-43* mouse models^[40-42]. Therefore, these pig models of ALS have a great value in studying the pathogenesis mediated by cytoplasmic mutant *TDP-43* or intranuclear *SOD1*.

Pig models of Huntington's disease

Huntington's disease (HD) is an autosomal dominant and age-dependent neurological disorder characterized by motor dysfunction, cognitive decline, and psychological disturbance. Pathologically, HD is characterized by selective neurodegeneration, which preferentially occurs in the striatum. Most HD patients develop symptoms in middle age, and the symptoms worsen with age with patients usually dying 10-15 years after symptom onset^[43]. HD results from a monogenetic mutation of a CAG repeat expansion in the exon1 of the gene Huntingtin (*HTT*). *HTT* is a multifaceted protein that is expressed ubiquitously and has numerous roles^[44]. CAG repeat expansion (> 36 CAGs) in the *HTT* gene is translated to a polyglutamine (polyQ) expansion that causes *HTT* to misfold and aggregate in the brain. HD transgenic mice and HD-KI mice have been widely used, but their brains do not display the selective and striking neuronal loss seen in human HD patients^[45].

In 2001, a transgenic pig model for HD was produced by pronuclear microinjection. However, the development of behavioral and neuropathological symptoms of HD in this transgenic pig model remains unclear^[46,47]. In 2010, researchers used SCNT to successfully establish a transgenic HD pig model expressing N-terminal mutant *HTT* (1-208 amino acids) with 105Q. This pig model showed apoptosis in the brain and died postnatally. However, mice expressing the same transgene did not produce the brain pathology seen in pigs^[48]. Later, another group used lentiviral transduction of pig embryos to establish a transgenic minipig model of HD expressing N-terminal mutant huntingtin (1-548 aa) under the control of human *HTT* promoter. However, this pig model did not develop motor deficits at up to 40 months of age, although mutant *HTT* mRNA and protein fragments were detected in the brain and peripheral tissues^[49].

It is apparent that the phenotypes of transgenic HD pig models are dependent on the expression levels of transgenic N-terminal mutant *HTT*. It is important to create a pig model that expresses full-length mutant *HTT* at the endogenous level. With the development of CRISPR/Cas9 technology, precise gene editing of various animal species becomes possible^[33], especially for the generation of large animal models^[32]. To overcome the shortcomings of the transgenic pig model of HD, Yan *et al.* first used CRISPR/Cas9 to insert a large CAG repeat (150 CAGs) into the pig *HTT* locus in fibroblast cells, and then used SCNT to generate a HD knock-in pig model^[50]. The brains of this pig model showed severe and preferential neurodegeneration in the medium spiny neurons in the striatum, an important pathological feature in HD patients. More importantly, the HD pig models displayed dance-like symptoms and breathing difficulties, which were similar to the symptoms in HD patients. Further, the pathogenic and neurologic features of HD pigs can be stably passed to offspring, enabling the establishment of a large animal model of HD for mechanistic study and drug screening.

Pig models of Alzheimer's disease

The incidence rate of Alzheimer's disease (AD) is increasing year by year with aging. Its early neurological symptoms are mainly memory loss and behavioral changes, and, in the late stage, the patients will have cognitive impairment, which severely affects daily life^[51]. AD is usually divided into familial AD (FAD) and sporadic AD (SAD) according to different pathologies. Only about 5% of AD cases are FAD and are caused by mutations in β -amyloid precursor protein (APP), presenilin 1 (*PS1*), and/or presenilin 2 (*PS2*). Nearly 95% of patients with AD are classified as SAD, which is caused by a combination of genetic factors and environmental risk factors without documented familial history of AD^[52]. The deposition of β -amyloid ($A\beta$) and hyperphosphorylation of Tau are the major pathological hallmarks, with other pathophysiologic changes including neuroinflammation, oxidative stress, and abnormal lipid metabolism. In addition to $A\beta$ and Tau, apolipoprotein E4 (*APOE4*) and coulomb-receptor expressed on myeloid cells 2 (*TREM2*) are considered to be the risk factors^[53]. Various mouse models of AD have been developed to mimic the symptoms of AD. However, due to the complexity of the neuropathology spectrum of AD, none of the available mouse models truly recapitulate the full spectrum of AD neuropathology, which includes $A\beta$ deposition, synapse loss, inflammation, tau hyperphosphorylation, and neurofibrillary tangle formation^[54]. To model the characteristics of AD in more human-like species, researchers injected $A\beta$ oligomers into the lateral ventricle of macaques, which diffused into the brain and accumulated in several regions associated with memory and cognitive functions. They found that oligomer injections induced AD-like pathology with neurofibrillary tangle formation in the macaque brain, which was not found in small animal models^[55]. Other researchers also used viral delivery of human 4R-tau to generate a tau-based rhesus monkey model of Alzheimer's disease^[56]. However, due to the long reproductive cycle of monkeys and immature cloning technology, it was difficult to obtain a large group of monkey models of AD through transgenic methods. Therefore, the establishment of transgenic pig models of AD is needed.

In 2009, Kragh *et al.* tried to develop a pig model of Alzheimer's disease by expressing AD-causing dominant mutation *APP^{sw}*. The transgene consisted of the cDNA of the neuronal variant of the human *APP* gene with the Swedish mutation. However, no disease phenotype was reported, although it was predicted that accumulation of the $A\beta$ peptide in the brain might develop at the age of 1-2 years^[57]. The same group also generated a transgenic miniature pig model expressing a cDNA of the AD-causing gene *PSEN1M146I* driven by an enhanced human UbiC promoter. However, no phenotypic data have been published yet^[46,58]. To induce the neuropathology of the increased intraneuronal $A\beta$ plaque formation, this group combined the mutation of *PSEN1* and *APP* together to generate double transgenic Göttingen minipigs that carry one copy of a human *PSEN1* cDNA with the Met146Ile (*PSEN1M146I*) mutation and three copies of a human *A β PP695* cDNA with the Lys670Asn/Met671Leu (*A β PPsw*) double mutations. Their strategy successfully generated a pig model with an intraneuronal accumulation of $A\beta_{42}$ in the brain between the age of 10 and

18 months, which may represent an early event in the pathogenesis of AD^[59]. In 2017, another group used a retroviral multi-cistronic vector to generate an AD transgenic pig carrying three AD-related genes with a total of six well-characterized mutations: *hAPP* (K670N/M671L, I716V, and V717I), *hTau* (P301L), and *hPS1* (M146V and L286P). They confirmed that transgenes were expressed at especially high levels in the brain. The levels of A β -40/42, total Tau, and GFAP were high in the brains of these transgenic animals as well. They proposed that more tests are needed in the future to find out if these pigs have age-dependent phenotypes of AD^[60].

Pig models of Parkinson's disease

Parkinson's disease (PD), characterized by slowness of movement, limb stiffness, and tremors, is the second most common neurodegenerative disorder in the world. PD patients may also have issues such as cognitive issues, depression, anxiety, olfactory loss, and gastrointestinal disorder. The motor symptoms of PD are caused by the death of dopaminergic neurons in the substantia nigra^[61]. Loss of dopamine neurons causes a drop in dopamine levels in the striatum, which leads to disrupted motor control^[62]. Many mutations or variants in a number of genes, such as α -synuclein (SNCA), leucine-rich repeat kinase 2 (*LRRK2*), ten-induced kinase 1 (*PINK1*), arkin (*PRKN*), and protein deglycase (*DJ-1*), are found to increase the susceptibility to PD and have been used to create genetically modified animal models of PD^[62,63]. However, many mouse models do not recapitulate the selective and progressive neurodegeneration seen in PD^[64,65]. Although non-human primate models of PD have been established for investigation^[66,67], it is difficult to establish a cohort of PD monkey models. Some teams thus explored the generation of pig models to study the neurological phenotypes of PD.

Yao *et al.* used TALENs combined with SCNT and embryo transfer to generate *DJ-1* KO piglets by disrupting the *PARK7* gene to model the phenotype of PD. Unfortunately, the piglets all died due to cloning defects, although *DJ-1* protein was successfully repressed in all the detected tissues^[68]. Another group used CRISPR/Cas9 combined with SCNT to generate *PARK2* and *PINK1* double-gene KO pigs. However, as with mouse PD models, no phenotypic symptoms of PD were observed in the seven-month-old live mutant pigs^[69]. In 2016, Wang *et al.* generated a PD pig model using CRISPR/Cas9 system by simultaneously targeting three distinct genomic loci, *Parkin/DJ-1/PINK1*, in Bama miniature pigs. However, the piglets remained healthy with a normal growth rate, and no typical symptoms of Parkinson's disease were observed in the 10-month-old live mutant pigs in this study^[70].

BASE EDITING USED IN PIG MODELS

Although the CRISPR/Cas9 system has been widely used to facilitate genome editing, it could induce random insertions or deletions (indels) through error-prone NHEJ rather than the error-free HDR^[35]. As a result, indels are obtained much more frequently at targeting sites than single-nucleotide substitutions. However, most human neurological diseases are induced by point mutations, rather than indels^[71], which emphasizes the importance of the application of the genome-editing technique of base editing in the establishment of animal models of human neurological disease.

Base editing is a genome-editing technique that generates mutations at single-base resolution^[72-74]. All four transition mutations, namely C to T, G to A, A to G, and T to C, can be inserted into the genome with the available CRISPR/Cas base editors (BEs). The cytosine base editor (CBE) can insert a C-G to T-A mutation, while the adenine base editor (ABE) can alter an A-T base pair into a G-C pair. In RNA, conversion of A to inosine (I) is also possible with the RNA base editor (RBE)^[75].

The above advanced technologies have already been used to generate many genome editing models, especially in small animals and plants, such as mouse^[76,77], rat^[78], rabbit^[79], sheep^[80], rice^[81], and wheat^[82]. Some groups have also succeeded in applying this tool to large animals^[83,84].

As for pigs, Li *et al.* first established pig models created via BE3, which separately targeted the *TWIST2* gene and the *TYR* gene^[85]. These pig models were able to reproduce the phenotypes of human diseases, which indicates that base editing systems provide a safer and more efficient approach to generating pig models that can precisely mimic point mutations of human diseases. Another study also indicated that using base editing technology was able to precisely introduce three gene (*GGTA1*, *B4galNT2*, and *CMAH*) base conversions into the pig genome with high efficiency^[71]. In summary, there is enormous potential for establishing pig disease models of neurological disease through base editing because of its significant advantages compared with the traditional CRISPR/Cas9 system.

POTENTIAL LIMITATIONS OF USE OF PIG MODELS

Currently, pig models for neurodegenerative diseases provide considerable support for the analysis and treatment of such diseases in humans. In general, pig models have great potential to advance the study of human neurodegenerative diseases, from pathogenesis research to the development of drugs, and even as donors of tissues and organs.

In addition, while pig disease models have greatly accelerated advances in studying genetic diseases and testing drugs and treatments, there are still some problems. First, pigs require more space than rodents in animal facilities and, thus, higher maintenance costs. Second, due to their large size, surgical operations need to be performed by trained personnel, and because its brain is wrapped in a thick skull, the collection of brain tissue requires a high degree of proficiency of the operator, which increases the experimental cost to a certain extent. Third, because of their large size, behavioral tests will be more difficult. However, at present, various behavioral studies of pigs have been gradually improved, for instance, learning and memory study using novel object recognition tests; anxiety and depression measurement using open field^[86]; neuropsychological screening for executive function, anxiety, willingness to explore a new environment, and locomotion using the open field test^[87]; and motor ability measurement using a 3D kinematic gait analysis system^[87].

CONCLUSION

A critical step in studying neurological diseases is to establish suitable animal models. Due to the complexity of neurological diseases, such as AD and PD, as well as the species differences between mice and humans, selective and overt neurodegeneration is not well modeled using mouse models. Pig models have great potential in modeling neurological diseases due to their close resemblance to the human nervous system, and several genetically modified pig models have been established for investigating neurodegenerative diseases [Table 1].

Pigs have very similar brain structure and function to humans. More importantly, pigs have sulci and gyri, and their brain volume is similar to that of humans, offering advantages over small animals for studying important brain diseases. Given their short reproductive cycle (5-6 months of sexual maturity) and multiple litter sizes (average of 7-8 piglets) as well as the availability of techniques for generating specific models of human diseases, pigs also have distinct advantages over non-human primates. Pigs can also be ethically used for translational research. For example, scientists and doctors recently successfully transplanted a pig heart into a patient with end-stage heart disease^[88]. This work opened up a new avenue in the study of xenotransplantation.

Table 1. Examples of neurodegenerative disease pigs described in this article

Pig models	Genes	Editing type	References
ALS pig	<i>SOD1</i>	TG	[38]
ALS pig	<i>SOD1</i>	TG	[9]
ALS pig	<i>TDP-43</i>	TG	[39]
HD transgenic pig	<i>HTT</i>	TG	[47]
HD transgenic pig	<i>N-mHTT(105Q)</i>	TG	[48]
HD transgenic pig	<i>HTT(1-548)</i>	TG	[49]
HD KI pig	<i>HTT</i>	KI	[50]
AD transgenic pig	<i>APP^{sw}</i>	PM	[57]
AD transgenic pig	<i>PSEN1(M146I)</i>	TG	[58]
AD transgenic pig	<i>PSEN1, APP</i>	PM	[59]
AD transgenic pig	<i>hAPP, hTau, hPS1</i>	PM	[60]
PD pig	<i>PARK7</i>	KO	[68]
PD pig	<i>PARK2, PINK1</i>	M-KO	[69]
PD pig	<i>Parkin, DJ-1, PINK1</i>	M-KO	[70]

The table lists genes that have been changed using TG, KO, M-KO, PM, or KI. TG: Transgenic; KO: knockout; M-KO: multiplex knockout; KI: knock-in; PM: point mutation (by HDR); ALS: amyotrophic lateral sclerosis; HD: Huntington's disease; AD: Alzheimer's disease; PD: Parkinson's disease.

The pig models can also be used for preclinical evaluation of stem cell therapy, gene therapy, and drug screening because their body size and metabolism are closer to humans than other species. Their relatively fast breeding and reproduction would provide a sufficient number of animals for evaluation of the therapeutic effects of drugs and other means. Considering the advanced gene editing tools available, we believe that genetically modified pig models will play a more important role in the studies of age-dependent neurological diseases in the future.

DECLARATIONS

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Authors' contributions

Wrote the review paper: Li C, Li J

Revised manuscript: Li S, Yan S

Conceived and designed experiments: Yan S, Lai L

All authors read and approved the final manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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