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INVITED REVIEW

# Animal modelling for inherited central vision loss

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## Abstract

**Disease-causing variants of a large number of genes trigger inherited retinal degeneration leading to photoreceptor loss. Because cones are essential for daylight and central vision such as reading, mobility, and face recognition, this review focuses on a variety of animal models for cone diseases. The pertinence of using these models to reveal genotype/phenotype correlations and to evaluate new therapeutic strategies is discussed. Interestingly, several large animal models recapitulate human diseases and can serve as a strong base from which to study the biology of disease and to assess the scale-up of new therapies. Examples of innovative approaches will be presented such as lentiviral-based transgenesis in pigs and adeno-associated virus (AAV)-gene transfer into the monkey eye to investigate the neural circuitry plasticity of the visual system. The models reported herein permit the exploration of common mechanisms that exist between different species and the identification and highlighting of pathways that may be specific to primates, including humans.**

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## The importance of cones for vision

Human vision is based on three systems of photosensitivity: (1) rod photoreceptors, (2) cone photoreceptors, and (3) melanopsin-expressing retinal ganglion cells. Together, these photosensitive cells cover 12 log of light intensity units [1]. Rod photoreceptor cells are responsible for the detection of stimuli in dim light, whereas cone photoreceptor cells transmit visual information in bright light conditions. Melanopsin-expressing retinal ganglion cells are also sensitive to bright light, and are implicated in non-image-forming visual functions such as circadian synchronization or pupillary light reflexes. Cones also provide colour vision and high visual acuity, allowing precise tasks such as reading or facial recognition. Phototransduction occurs in the long process of photoreceptor cells, termed the outer segment (OS), that is linked to the cell body by the inner segment (IS), which contains mitochondria. Without neglecting the heavy consequences of rod loss for vision (night blindness, for example), loss of the cone system is dramatic in our highly technological world, where reading and visual communication are extremely important for social interactions. Moreover, in patients suffering from rod deficiency, a secondary death of cones is observed which definitely worsens their quality of life. To date, no molecular therapy has been successful in stopping cone degeneration, and physicians can only encourage patients to adapt to their vision loss or propose diets that might slow the process in certain conditions [2]. Thus, the study of cone degeneration mechanisms and the examination of innovative therapeutic strategies are a major field of research in ophthalmology.

Very good reviews on retinal dystrophies, including cone dystrophies, were recently published and we encourage the reader to refer to these works for an exhaustive documentation [3–5]. The present review focuses mainly on genetically modified models and summarizes the naturally occurring models for pronounced cone dystrophies (Table 1).

## In vitro modelling of cone photoreceptors

The development of induced pluripotent stem cells (iPSCs) has brought new opportunities to dissect *in vitro* the molecular mechanisms of cone degeneration. Fibroblasts or blood cells may be reprogrammed using four key genes (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) to acquire an embryonic stem cell-like stage [6] which is characterized by pluripotency. These cells, or embryonic stem cells, can then be differentiated into cell types of interest such as photoreceptor cells [7–10]. The *in vitro* generation of cones from patients with specific cone disorders could thus provide an accurate model to study

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Table 1. Summary of the animal models for cone dystrophies. The table lists the animal models described in the review, compiling the species, the name of the gene that was modified, the method of generation of the model, and the human disease targeted

| Model | Species | Gene | Method of generation | Human condition | Reference |
|---|---|---|---|---|---|
| *pTol2:3.2 gnat2-RETGC-1 E837D R838S* | *Danio rerio* | *RETGC-1* | Embryo injection and transposase system | CORD6 | [27] |
| *rd/rd* Rhode Island Red chicken | *Gallus gallus domesticus* | *GC1* | Spontaneous mutation | LCA | [35] |
| *Rpe65*$^{R91W/R91W}$ | *Mus musculus* | *Rpe65* | ES homologous recombination | LCA | [40] |
| *Rpe65*$^{-/-}$ | *Mus musculus* | *Rpe65* | ES homologous recombination | LCA | [42] |
| *Rpe65*$^{rd12}$ | *Mus musculus* | *Rpe65* | Spontaneous mutation | LCA | [41] |
| *Lrat*$^{-/-}$ | *Mus musculus* | *Lrat* | ES homologous recombination | LCA, RP | [128] |
| *Nrl*$^{-/-}$ | *Mus musculus* | *Nrl* | ES homologous recombination | ESCS syndrome | [53] |
| *rd7* | *Mus musculus* | *Nr2e3* | Spontaneous mutation | ESCS syndrome | [55] |
| *Rpe65*$^{-/-}$*;Nrl*$^{-/-}$ | *Mus musculus* | *Rpe65;Nrl* | Breeding of single knockout | Macula region | [63,65] |
| *Rpe65*$^{R91W/R91W}$*;Nrl*$^{-/-}$ | *Mus musculus* | *Rpe65;Nrl* | Breeding of single knockout | Macula region | [66] |
| *Gucy2e*$^{-/-}$*;Nrl*$^{-/-}$ | *Mus musculus* | *Gucy2e;Nrl* | Breeding of single knockout | Macula region | [71] |
| Sudanian grass rat | *Arvicanthis ansorgei* | | MNU and light-induced toxicity | Macular degeneration | [51,52] |
| Nile grass rat | *Arvicanthis niloticus* | | | Macula region | [49] |
| Awassi sheep | *Ovis orientalis* | *CGNA3* | Spontaneous mutation | ACHM | [72] |
| Pig *TgP347L*, Pig-*Tg N1Pet* | *Sus scrofa* | *RHO* | Pronuclear injection and homologous recombination | RP | [79] |
| Pig *TgP23H*, line *53-1* | *Sus scrofa*, NIH minipig | *RHO* | Somatic cell nuclear transfer | RP | [80] |
| Pig *ELOVL4 – 790e794delAACTT* | *Sus scrofa* | *ELOVL4* | Pronuclear injection and homologous recombination | STDG3 | [91] |
| Pig *ELOVL4 – Y270terEYFP* | *Sus scrofa* | *ELOVL4* | Somatic cell nuclear transfer | STDG3 | [91] |
| Pig *GUCY2D -E837D;R838S* | *Sus scrofa* | *GUCY2D* | Lentiviral-directed transgenesis | CORD6 | [93] |
| *cmr1, cmr2, cmr3* | *Canis lupus* | *BEST1* | Spontaneous mutation | Macular dystrophy | [95] |
| *XLPRA1, XPLRA2* | *Canis lupus* | *RPGR* | Spontaneous mutation | XLRP | [96] |
| *crd* | *Canis lupus* | *NPHP4* | Spontaneous mutation | LCA | [98] |
| *crd* | *Canis lupus* | *RPGRIP1* | Spontaneous mutation | LCA | [101] |
| *crd2* | *Canis lupus* | *NPHP5, IBQ1* | Spontaneous mutation | LCA, SLSN | [100] |
| *crd3* | *Canis lupus* | *ADAM9* | Spontaneous mutation | CORD9 | [106] |
| Achromatopsia Alaskan Malamute | *Canis lupus* | *CNGB3* | Spontaneous mutation | ACHM | [110] |
| Achromatopsia German Shorthaired Pointer | *Canis lupus* | *CNGB3* | Spontaneous mutation | ACHM | [110] |
| Retinal transduced squirrel monkey | *Saimiri sciureus* | *L-OPSIN* | Somatic retinal transfer | Trichromacy recovery | [111] |

cone imbalance or cone death and to screen therapeutic compounds. Although the creation of retina-like tissue derived from iPSCs allows analysis of photoreceptor generation [11–13], the production of human cones is still poor and the process does not recapitulate the formation of the macula or the subsequent synaptic connecting network. In consequence, the first disadvantage of these *in vitro* models is that so far, no culture system enables study of the mechanisms of human macular degeneration, especially when this loss is due to a deficiency of neighbouring cells such as Müller and retinal pigmented epithelial (RPE) cells. Special effort to adapt cultures of multiple cell types would thus be needed to mimic *in vivo* conditions. Second, in some cone dystrophy cases, the time necessary to obtain cone degeneration might not be perfectly reproducible *in vitro* as the onset varies from shortly after birth (achromatopsia) to 70 years of age (as in some patients affected by the Stargardt diseases, see the review by Roosing *et al* [4]). Third, even if this *in vitro* model could be useful to screen for the potential of specific compounds to rescue cones, it could not fully validate the efficiency of the technological transfer *in vivo* or assess for the global effect induced in the long term.

## Non-mammalian cone-rich models

Retinal research benefits from a wide panel of animal models. Many gene deficiencies implied in visual defects affect only vision which, at least in laboratory conditions or domestic breeding, is not required for survival. Thus, numerous animal models have been described following spontaneous mutations of genes also involved in human retinal disorders [3,14,15]. Most rodents, like most mammalians, are dichromatic and have only two types of cones, sensitive to medium (M-cone) and short (S-cone) wavelengths, in contrast to primates and humans, who have three types of cones: short (blue), medium (green), and long (L-, red) wavelengths (Figure 1). Interestingly, birds, reptiles, and fishes are also at least trichromatic or even tetrachromatic, which gives alternatives to researchers for modelling cone disorders.

Among fishes, the model of choice selected for development and physiopathology studies is the zebrafish, thanks to its widely known genetics [16], even though several cone physiological studies have been performed using carp. In addition to carrying three cone types, the zebrafish has a fourth UV-type of cone, as well

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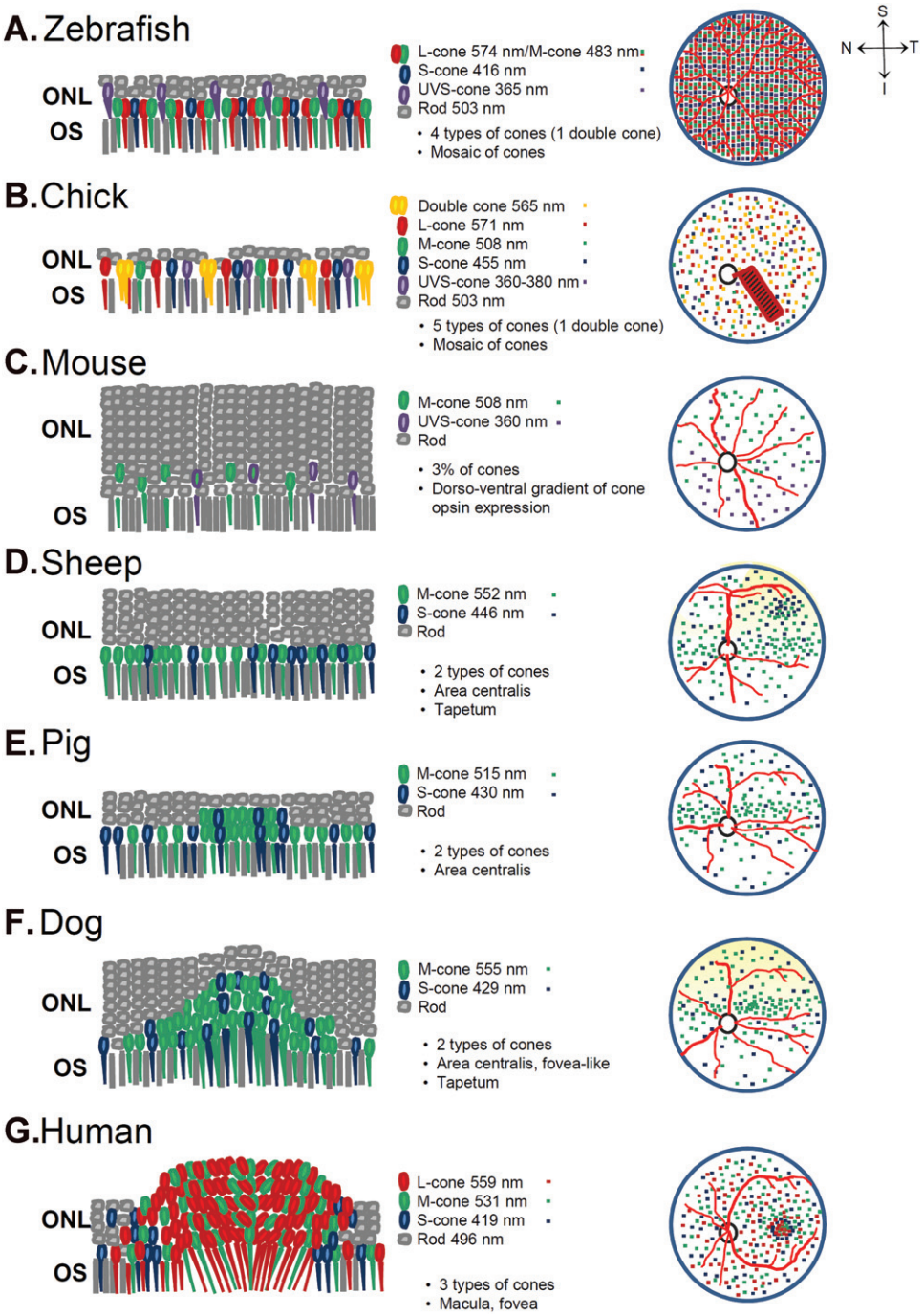


**Figure 1.** Schematic representation of cone distribution in the central retina of different species. Both sections of the outer nuclear layer and visualization of the eye fundus were schematically represented to highlight the specific cone distribution of the retina of zebrafish, chick, mouse, sheep, pig, dog and human. Photoreceptor types are arbitrarily coloured to represent the different categories of cones (L, M, S, and double cones) and rods. The wavelength of each photopigment is also indicated. (A) In zebrafish, in addition to S- and UV-cones, a double cone is observed with fusion at the level of the IS of two OSs containing either L- or M-opsin. The four classes of cones are then laid out in a regular mosaic pattern. (B) The chick retina contains five types of cones comprising a double cone (in yellow) as well. The different types of cones are homogeneously arranged in the retina. A particular vascular extension, the pecten (red rectangle), is apposed at the inner part of the retina. (C) The mouse retina is composed of only 3% cones, distributed throughout the retina. A dorso-ventral gradient of cone expression is observed, with S-opsin mainly expressed in the inferior hemisphere and M-opsin in the superior hemisphere. However, both opsins are observed in single cells in the overlapping gradient. (D) The sheep retina includes two types of cones, with higher densities of cones in the central streak and in the dorso-temporal region. S-cones are enriched in this particular peripheral region. Sheep have a tapetum, a membrane reflecting the light in the superior hemisphere (yellow area) except in the dorso-nasal periphery. (E) The pig retina features two types of cones, with densities higher in the central streak. (F) The dog retina is also characterized by two types of cones and a central streak, but recently a fovea-like region was identified with an increased number of cones and a longer OS. Dogs also have a tapetum in the superior hemisphere (yellow area). (G) The human central retina is characterized by a region with exclusively cones named the fovea, containing mainly L- and M-cones. S-cones are distributed in the perifovea region and in the periphery. ONL: outer nuclear layer; OS: outer segment; black circle: optic nerve head; red lines: vessels; S: superior; I: inferior; N: nasal; T: temporal. Source: refs 32, 72, 94, and 112–127; http://www.cvrl.org/database/text/intros/introdens.htm

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as calyceal processes, which are membrane extensions from the apical side of the IS surrounding the basal part of the OS [17] and are described in primate cones but are absent in mouse cones [18]. Several external factors such as light exposure have been used to induce and study photoreceptor degeneration [19–22]. In addition to screening of mutagenized zebrafish stocks [23,24], gene editing tools offer many possibilities to engineer zebrafish models for specific genes [25]. However, the major limitation of zebrafish models is the capacity of the retina to regenerate, which may affect the parallelism with patients [26]. For example, a dominant mutant allele of the guanylate cyclase 2D (*GUCY2D*) gene under the control of a cone promoter was used to perform transgenesis with a transposase system [27] in order to model the phenotype of cone–rod dystrophy (CORD6) patients [28–30]. In these families, the first symptoms described are decreased visual acuity, followed by loss of colour discrimination during the first decade. In the transgenic GUCY2D mutant zebrafish model, no early changes in visual function (optokinetic response) were detected, despite decreased staining of cone and rod markers in both larva and adults. Whether the regenerative properties of the zebrafish retina impaired the progression of the disease remains unknown.

Chickens are an alternative model suitable for cone studies, thanks to the high content of cones in their retinas [31,32]. Moreover, the embryonic stages are easily accessible and the large size of the chick eye during development makes them an appreciated model for development studies [33,34]. No transgenic models for retinal diseases have been engineered but the spontaneous model of *GC1* gene loss of function [35] was used to demonstrate the efficiency of gene replacement using lentiviral vectors [36]. A retinal detachment model was also produced by subretinal injection of saline or hyaluronic acid to study cone survival in this paradigm [37]. However, the organization of the chicken vasculature is different from that in humans as their retina is avascular but with a highly glial and vascular structure called the pecten, which is localized in the vitreous of the eye [38,39]. Thus, the retina's morphology appears different between birds and mammals, as probably is cone physiology.

## Novel perspectives on rodent modelling of cone-rich retina

Mouse and rat animal models of cone dystrophy face a major limitation due to the absence of the highly specialized central region found in the monkey and human retina forming the macula and the fovea (Figure 1). As murine and rat retinas have only 3% of cone-type cells spread within rods, they are more adapted for modelling the peripheral primate retina, where the blue-sensing cones are mainly distributed. For example, in the $Rpe65^{R91W}$ knock-in mouse model where a mutation encountered in patients was substituted to the murine wild-type codon [40], S-cones were rapidly affected and degenerated during the first months of life. The cone loss was even more rapid in the case of complete ablation of the gene ($Rpe65^{-/-}$ or the $Rpe65^{rd12}$ spontaneous model [41,42]) or in other chromophore-deficient models ($Lrat^{-/-}$ [43]). This early degeneration of S-cones is consistent with the phenotype of patients with RPE65 p.R91W, who endure early blue cone dysfunction in addition to rod desensitization [44]. Interestingly, Samardzija *et al* demonstrated that in cases of limited levels of chromophore in mice, rods compete with cones for chromophore loading [45]. This observation could explain why the peripheral human blue cones (equivalent to the murine S-cones) might be more affected than foveal cones in these patients deficient in RPE65.

In order to find rodent retinas enriched in cones, some researchers have looked further amongst diurnal animals, which are in general richer in cones than nocturnal ones [46]. Examination of the retinas of *Arvicanthis ansorgei* and *A. niloticus*, the Sudanian and Nile grass rats, respectively, revealed a ten-fold higher number of cones compared with *Mus musculus* [47–49]. As these animals are genetically closer to mouse than the ground squirrel (90% cones) [50], research using them can benefit from many tools developed for mice (antibodies, databases, maintenance, etc). The 30% of cones composing the *A. ansorgei* retina were thus proposed to offer a better model for the human macula. This model was used to demonstrate the separate cone death succeeding rod death in the case of MNU-induced toxicity, as well as the high resistance of cones to stress [51,52]. However, no models for specific gene dysfunctions have been described yet, limiting the studies of *Arvicanthis* retinas to the use of external damaging factors.

In contrast, the deep understanding of the genetics of retinal development in mice created opportunities to generate mouse models deprived of rods and enriched in cone-like cells. The genetic ablation of the Nrl transcription factor in the $Nrl^{-/-}$ mouse impairs rod development and maintains the precursor in the default cone differentiation pathway [53,54]. Similar results were observed in *rd7* mice, where a retrotransposon is inserted in the *Nr2e3* gene, a downstream target of Nrl. In the retina of *rd7* mice, hybrid photoreceptors called 'cods', displaying many characteristics of S-cones, are present but no enhanced S-cone system is detected by electroretinogram (ERG) recordings [55–59]. In humans, NRL gene variants have been identified in patients suffering from an enhanced S-cone sensitivity syndrome. Studies in the $Nrl^{-/-}$ mouse have enabled a better understanding of the origin of supranormal photopic activities of patients but have also revealed the phagocytosis defect of $Nrl^{-/-}$ cone OSs, which seems consistent with imaging reported in patients [60]. Moreover, transient and partial cone degeneration was observed between 1 and 4 months in $Nrl^{-/-}$ mice, followed by the long-term persistence of remaining cones [61]. The circulating retinal current of the young adult $Nrl^{-/-}$ retina enriched in cone-like cells is less demanding of oxygen compared with a WT rod-rich retina [61,62]. Hyperoxic conditions

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are thus created and are thought to induce a remodelling of the *Nrl*$^{-/-}$ retinal architecture with Müller cell activation, RPE atrophy, and loss of vasculature. Consistent with this hypothesis is the work on the *Rpe65*$^{-/-}$;*Nrl*$^{-/-}$ double knockout mouse that showed a role for the chromophore in the formation of rosettes in the *Nrl*$^{-/-}$ background, because the absence of 11-*cis*-retinal in the *RPE65*$^{-/-}$ background allows recovery of a normal layered retina [63–65]. However, these mice have no visual function, due to the absence of a chromophore, and are thus of limited interest. Samardzija *et al* proposed the use of the *Rpe65*$^{R91W}$;*Nrl*$^{-/-}$ mouse as an alternative [66], and indeed the persistence of a 10% wt level of RPE65 protein as well as its remaining residual activity allows efficient suppression of the formation of rosettes while maintaining a recordable retinal activity [66]. Retinal markers and functional tests also revealed that the *Rpe65*$^{R91W}$ background preserves second- and third-order neurons (horizontal and retinal ganglion cells) that are usually affected secondarily in the *Nrl*$^{-/-}$ background. The vasculature was also rescued and this renders the *Rpe65*$^{R91W}$;*Nrl*$^{-/-}$ mouse a reliable all-cone retina model with a preserved retinal architecture. In this complex genetic background, cone loss can be studied in acute or slow degeneration processes using MNU administration or specific ablation of phosphodiesterase (PDE) genes [66].

Other gene defects have been examined in this *Nrl*$^{-/-}$ cone-only background and highlight the efficiency of cone rescue after gene replacement strategies. For example, Du *et al* [67] demonstrated the efficiency of intra-vitreous injection of the AAV8 (Y447, 733 F) vector to target cones and restore vision in *Cnga3*$^{-/-}$;*Nrl*$^{-/-}$ mice. The AAV8 (Y447, 733 F) capsid is a genetically modified capsid 8 protein which can cross the retina from the inner side and reach the outer part of the retina to transduce photoreceptor cells. The authors thus validated a less damaging approach for vector delivery to cones than subretinal injection [67]. Similarly, Boye *et al*, after validation in multiple guanylate cyclase-deficient murine models of Leber congenital amaurosis [68–70], also demonstrated cone rescue in the *Gucy2e*$^{-/-}$;*Nrl*$^{-/-}$ model after subretinal injection of AAV8 (Y733)-hGRK1-*Gucy2e* or AAV5-hGRK1-*Gucy2e* vectors [71]. Taken together, these results demonstrate how fundamental research on the development and physiology of the rodent retina has opened new perspectives for disease modelling and therapy development.

## Naturally-occurring cone dystrophy in sheep

The sheep retina, although having no macula, shows two cone-enriched regions: a streak in the central part of the retina and a dorso-temporal small area (Figure 1D). The central region is termed the area centralis and is composed of two cone types [short wavelength (S-cone) and medium/long wavelength cones (M/L-cones)], but S-cone density is particularly high in the dorso-temporal region. In addition, a tapetum, a layer located behind the retina, covers the dorso-temporal region with an extension towards the nasal area. This tapetum enhances the chance of photon capture by the photoreceptors by reflecting photons back to the retina. A naturally-occurring day blindness was observed in the improved Awassi breed [72] and was associated with a mutation in the gene encoding the α subunit of the cone photoreceptor cyclic nucleotide-gated (CNGA3) [73]. Lambs showed an almost normal rod function, whereas ERG recorded in photopic conditions (in which the animal is light-adapted and receives stimuli in presence of an illuminated background) revealed a diminished amplitude of cone responses. The pupil light reflex was also impaired in the daytime. The presence of a well-preserved retina during the first months of the disease renders these animals an interesting model for a gene therapy treatment to recover cone function at different time points through the course of disease. Recently, Banin *et al* [74] restored cone function in these animals by AAV2/5 gene transfer.

## Transgenic pigs to study retinal dystrophies

The pig retina also contains an area centralis composed of around 30% cones that express M/L-opsin and S-opsin (Figure 1E). Novel therapeutic approaches can be tested using this model since the pig, similarly to primates and humans, presents a fully developed retina at birth, and benefits from an immune system comparable to the human one. Moreover, the large size of the eye makes it particularly suitable for validating procedures that may be suited to use in humans. Among various strategies, pigs have been used to test retinal prosthesis implants [75], cell transplantation [76,77], and vector serotypes as well as promoter activity for gene therapy developments [78].

### Pig rod dystrophy models for studying cone degeneration

The first transgenic (Tg) pig to model a retinal dystrophy was generated by pronuclear microinjection to mimic a severe form of retinitis pigmentosa caused by the dominant rhodopsin (*RHO*) gene mutation p.P347L [79]. The 5'-upstream region of the human *RHO* gene served as a promoter for the human mutated allele. This work was then followed by the generation of TgP23H pigs [80] using stable-transfected pig fibroblasts, which then served for nuclear transfer. In this study, the transgene was controlled by the phosphoglycerate kinase promoter and the NIH miniature pig (SLA$^{C/C}$ haplotype) was used. Although these mutations affect specifically rod function and survival, these models are also interesting for cone studies as cone death is observed following rod loss. This secondary wave of photoreceptor death is hypothesized to be related to a deficit in a survival factor secreted by rods, the rod-derived cone viability factor

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(RdCVF [81]) and in nutrients supplied by the choroid, the main component probably being glucose [82]. Very recent work linked RdCVF and glucose by showing that this factor binds cones on basigin-1, favouring glucose intake [83]. This RdCVF deficit is one explanation for the consequence of rod death on cone survival but does not elucidate the whole mechanism governing cone death during the late phase of retinitis pigmentosa. In this context, the availability of large transgenic animal models of retinal dystrophies such as pigs is a very valuable improvement in studying the process of cone death and developing new therapies to inhibit this process.

In both TgP347L and TgP23H pigs, in which rod function is severely affected, cone activity is also altered and continuously declines during the course of the disease [79,80,84]. The TgP347L pig showed a decline of the retinal responses measured by ERG in photopic conditions at 4 and 87 weeks of age [79]. For the TgP23H pig, the first time point investigated was at 14 days of age in the most severely affected animals, and these showed an absence of the scotopic response (in which animals are dark-adapted and receive stimuli in a dark background in order to reveal rod function) [85]. The cone response had declined at 2 months and had decreased to 50% at 3 months of age [80,85]. The photopic response then remained at approximately 30% of the WT response until 18–24 months. Surprisingly, cone morphology, as evidenced by electron microscopy, was already altered during the retina's development at E10.5 and P3, despite normal recordings in photopic conditions obtained at P3 [86]. These two models clearly reveal that cone degeneration rapidly follows rod loss, indicating that cone protection needs to be performed at an early stage of rod dystrophies.

### Stargardt-like macular dystrophy type 3 in pigs

A juvenile form of macular degeneration termed Stargardt-like macular dystrophy type 3 (STGD3) is associated with mutations in the elongation of the very long-chain fatty acids-4 gene (*ELOVL4* [87]), which codes for an enzyme of the elongase family involved in long-chain fatty acid synthesis [88] and is mainly expressed in rods and cones [87]. The cones in the macula are particularly affected, in comparison to rods, by the g.790_794delAACTT deletion (resulting in the loss of 51 terminal amino acids); the p.Y270X mutation or the allele bearing a double single nucleotide deletion g.[789delT;794delT] also results in a dominant form of the disease [89,90]. A total of 1226 injections into pig pronuclei were performed with a construct expressing *ELOVL4* g.790_794delAACTT or a fluorescent protein (EYFP) fused at the C-terminus of the p.Y270X variant (p.Y270terEYFP). Gene expression in both constructs was controlled by the *RHO* Rho4.4 promoter (4.4 kb fragment upstream of the rhodopsin coding sequence, the exact origin not being indicated) [91]. The embryos were conveyed into 22 gilts, from which three transgenic animals were generated. Concerning the p.Y270terEYFP construct, a similar number of embryos were prepared, but with the nuclear transfer technique, and four pig lines were generated. The expression of the transgene appeared to be preferentially present in the cones (only one row of photoreceptors in the area centralis positive for EYFP in the outer part of the ONL). Nonetheless, the ERG recordings revealed that both rod and cone functions were altered in some animals at 14.5 months. These experiments show that the presence of truncated ELOVL4 proteins recapitulates different features of the STGD3 disease in pigs as it did in mice [88], thus proposing interesting models to study the pathophysiology of this disease.

### Production of a large cohort of transgenic pigs to study CORD6

CORD6 is the most frequent dominant cone dystrophy form in humans. The p.E837D and p.R838S variants of the *GUCY2D* gene product are associated with disease appearance [29,92]. By using a lentiviral vector encoding the mutated *GUCY2D* cDNA controlled by the cone arrestin promoter and injected in fertilized oocytes, Kostic *et al* [93] generated three cohorts of transgenic pigs. Around 50% of the offspring carried from one to six copies of the transgene. No abnormal behaviour or adverse health issues were observed in the piglets, despite integration of the transgene into the pig genome. A series of mobility tests based on vision were performed at different ages in parallel to optical coherence tomography (OCT) and ERG recordings to evaluate the morphological and physiological integrity of the retina. Interestingly, a wide range of response patterns to all these tests was described within the transgenic group. Although the response variability was high in the transgenic pigs, changes in the behaviour tests were observed in several transgenic animals and a significant alteration of transgenic cone activity was noticed by ERG in comparison to WT littermates. Moreover, histology revealed that all transgenic pig eyes had displaced cone nuclei that localized to the OS region, indicating a degenerative process. The overall results show that the *GUCY2D* gene bearing the two disease-causing variants p.E837D and p.R838S alters cone function and visual performance in photopic conditions, and induces a slow process of cone degeneration.

The variability observed among the transgenic animals is a consequence of the lentiviral transgenesis strategy used and may advantageously model the variability of the phenotypes observed in humans due to the incomplete penetrance of the disease. Moreover, the high percentage of the transgenic animal yield with the lentiviral vector (60% in this work) allows for the design of new experimental approaches to study the effect of dominant mutations.

## Dog models for cone dystrophies

Since 26 dog models of retinal dystrophies have already been characterized and described in a very well

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documented review [3], we will discuss here new data as well as dog models of cone dystrophy for which the gene mutation leads directly or predominantly to cone alteration. Like pigs, dogs possess an area centralis enriched in cones. However, detailed characterization of this region revealed that in a very small area, described as fovea-like, there is an elevation in the number of cone rows (from 1 to 3) and a decreased number of rod layers from 8 to 2–3 rows. In parallel, the RGC layer almost triples in this region, forming a dome around 200 µm wide. Moreover, cones and rods featured elongated segments [94]. All of these characteristics suggest that the dog retina contains a small fovea-like structure (Figure 1 F). These features render the dog retina very attractive to study gene mutations affecting mainly cone physiology and survival.

The study of two different dog models of cone dystrophies confirms that this fovea-like area behaves differently from other retinal regions. In Best's disease in humans, the macula is first affected, although the genetic mutation alters RPE function in general. In the *BEST1* dog model [95], the first lesion observed, corresponding to detachment of the retina from the RPE layer, appeared in the fovea-like region [94]. This observation suggests that the dog fovea displays a vulnerability to external environmental stresses similar to that in the macula of affected patients. In the dog model of human X-linked progressive retinal atrophy 2 (*XLPRA2*), variants in the *RPGR* gene lead to alteration of the structural cilium connecting the IS to the OS. In humans, mutations in the *RPGR* gene result in a wide range of phenotypes; nonetheless, the macula is often severely affected. Interestingly, in the dog model of *RPGR* alteration [96], the fovea is also the first area affected by the mutation, although the RPGR protein is altered in all cones. At 2 weeks of age, the cone number is already lower in the affected dog in comparison to WT but this number remains stable for 22 weeks. After this stable period, both cone and rod numbers decrease in the fovea, along the streak, and centrifugally.

These two examples provide evidence that the dog retina is of great value for studying cone diseases that affect the macula preferentially in humans. Several other naturally-occurring cone diseases have also been identified in dogs for which the identification of this fovea-like region may help to establish the first events appearing during these diseases.

### Cone dystrophies linked to cilium alteration

In standard wirehaired dachshund dogs, a mutation in the *NPHP4* gene, altering its product's ability to bind the RPGRIP1 cilium protein, was associated with an early-onset cone dystrophy [97,98]. Affected dogs showed an early-onset cone alteration at 5 weeks of age, evidenced by ERG in photopic conditions and using flicker stimulations [99]. In the cone rod dystrophy (*Crd2*) cone dystrophy dog, mutations in another member of this gene family, *NPHP5* (*IQCB1*), correlate with cone and rod abnormal segment formation, and progressive photoreceptor death [100]. Important variability was observed between dogs during the degenerative process. As expected, the mutation in the *RPGRIP1* gene found in the miniature longhaired dachshunds [101] is also linked to retinal ciliopathy. These dogs suffer from cone dysfunction starting at 7 weeks [102,103], but substantial variations in disease onset and severity have been observed in certain colonies [103,104] and not others [105]. Gene augmentation strategies with AAV2/5 and AAV2/8 have demonstrated the feasibility of re-establishing and maintaining cone function in RGRIP1-deficient dogs for at least 2 years.

Considered together, it is clear that these models recapitulate several retinal diseases observed in humans and enable the study of a network of proteins implicated in the mechanisms of ciliopathy in large animals. These results also reveal the existence of other genetic modifiers which remain to be identified.

### Cone dystrophy and extracellular matrix proteins

Glen of Imaal terrier dogs carry a deletion in the *ADAM9* gene resulting in the absence of exons 15 and 16 in the transcript which alters the protein's function [106]. Longitudinal studies (using different dogs) showed that the retinal activity declines with time, affecting both cone and rod functions. These data are in accordance with the progressive loss of the two photoreceptor populations. This animal model reproduces the major features observed in CORD9 patients and contrasts with the knockout mice that display subtle morphological changes within the RPE/photoreceptor interactions [107].

### Achromatopsia

In Alaskan Malamute dogs, a cone dystrophy was characterized by an early loss of day vision in bright light and normal vision in dim light [108]. Alteration of day vision was detectable between 8 and 12 weeks, and cone function was absent in young adults, as evidenced by red light stimuli. Rod function remained normal during the dog's lifespan. Electron microscopy analyses revealed that disorganization of the cone lamellar discs was already present in young dogs [109]. The high severity of the phenotype is correlated with total absence of the expression of *CNGB3*, which codes for the cone-specific channel subunit beta-3 [110]. A missense mutation in *CNGB3* was also associated with cone vision loss in German shorthaired pointer dogs. In these models, CNGB3 loss is causative for the day blindness observed in these dogs, similar to the majority of human achromatic patients [4].

## Creating trichromatic vision in the monkey

In the adult squirrel monkey strain (*Saimiri sciureus*), which has calyceal processes as do humans [18], the

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females have trichromatic vision (like humans), with cones expressing M, L, and S-opsins, whereas the males are dichromatic, with absence of the L-opsin. Mancuso *et al* [111] investigated whether the adult brain of a male monkey has enough plasticity to integrate the signal of a new chromophore to generate trichromatic vision, by transferring the L-opsin cDNA via AAV2/5 into male M-cones to extend their spectral domain to catch signals from longer wavelengths. This manipulation allowed the monkey not only to recognize a new subtle colour pattern among a grey background, but also to discriminate a blue-green (wavelength 490 nm) form from a red-violet background (wavelength 499 nm), a task which a non-treated animal failed. These results showed that the adult eye and brain can be reprogrammed for trichromatic vision and that the endogenous neural wiring is sufficient to integrate this new signal to augment environment perception. This new discrimination between 'red' and 'green' may be possible because not all red cones were transduced with the green opsin. The relative level of expression of green versus red opsin could also play a role in this acquired colour discrimination. This astonishing work opens up new perspectives for vision rehabilitation and new treatments via gene transfer not only for achromatopsia.

## Perspectives

Gene transfer has been proved to be efficient for the generation of transgenic animal models of different species. However, the main target for this approach is the study of the effect of genetic variants associated with dominant diseases. With the evolution of the gene editing technologies such as the CRISPR/Cas9 system, transgenesis will certainly be bolstered by models for recessive diseases enlarging the panel of molecular pathways available for research. So far, the different large animal models have mainly been used to make or affine genotype/phenotype correlations. In the future, the availability of several of these models will help to establish the molecular mechanism of diseases and allow us to better comprehend the biological differences between rodents and higher vertebrates, as well as prepare for the scale-up of new therapeutic approaches. In this context, large animals such as pigs, with a high rate of reproduction and less ethical concerns from the public, will have an advantage. The use of large animal models will definitively have an important societal impact for regenerative medicine and drug screening before launching a clinical trial.

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## Author contribution statement

CK and YA contributed equally to this review.

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