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How to cite

KATANAEV, Vladimir. Humanization for neurological disease modeling: A roadmap to increase the potential of Drosophila model systems. In: Animal models and experimental medicine, 2023, vol. 6, n° 3, p. 230–236. doi: 10.1002/ame2.12322

This publication URL: https://archive-ouverte.unige.ch/unige:169610
Publication DOI: 10.1002/ame2.12322

Received: 11 January 2023 | Accepted: 3 April 2023

DOI: 10.1002/ame2.12322

PRACTICE POLICY

Humanization for neurological disease modeling: A roadmap to increase the potential of *Drosophila* model systems

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Funding information
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Number: 31003A_175658

Abstract

Neuroscience and neurology research is dominated by experimentation with rodents. Around 75% of neurology disease-associated genes have orthologs in *Drosophila melanogaster*, the fruit fly amenable to complex neurological and behavioral investigations. However, non-vertebrate models including *Drosophila* have so far been unable to significantly replace mice and rats in this field of studies. One reason for this situation is the predominance of gene overexpression (and gene loss-of-function) methodologies used when establishing a *Drosophila* model of a given neurological disease, a strategy that does not recapitulate accurately enough the genetic disease conditions. I argue here the need for a systematic humanization approach, whereby the *Drosophila* orthologs of human disease genes are replaced with the human sequences. This approach will identify the list of diseases and the underlying genes that can be adequately modeled in the fruit fly. I discuss the neurological disease genes to which this systematic humanization approach should be applied and provide an example of such an application, and consider its importance for subsequent disease modeling and drug discovery in *Drosophila*. I argue that this paradigm will not only advance our understanding of the molecular etiology of a number of neurological disorders, but will also gradually enable researchers to reduce experimentation using rodent models of multiple neurological diseases and eventually replace these models.

KEYWORDS
disease modeling, *Drosophila*, humanization, neurological diseases

1 | INTRODUCTION

Neurological and neurodegenerative diseases plague our aging society, and have risen to become the leading cause of disability and the second leading cause of death worldwide.[1,2] Model organisms are widely used to study the molecular etiology of neurological diseases and to develop eventual treatments against them.[3] Despite the promise of non-vertebrate organisms such as *Drosophila melanogaster* in modeling neurological disease,[4,5] rodent models dominate this field of basic and translational research. In Switzerland alone, a country of ca. 8.7 million citizens, 0.9 million rodents (of which 0.7 million are mice) have been used for neurology disease-directed research in the period 2011–2021 (tv-statistik.ch/fr/statistiques-dynamiques). The insufficient adequacy of *Drosophila* disease models of human diseases is among the many reasons for the preferential use of mice versus fruit flies in neurology research. As elaborated here, this problem could be ameliorated by using the genetic humanization approach.

Traditionally, transgenesis techniques are used to build *Drosophila* models of human diseases.[6,7] For example, *Drosophila* models of Alzheimer's or Huntington's diseases typically rely on neuronal overexpression of human Aβ peptides[8,9] or human huntingtin,[10] respectively. Loss-of-function mutations in the *Drosophila* orthologs of the human disease-causing genes have also been analyzed to obtain insights into the (patho)physiological gene function, but may reveal unexpected phenotypes unrelated to the human pathology, as, for example, in the case of neurofibromatosis-1 (NF1) mutations in the fruit fly that lead to elevated reactive oxygen species (ROS) production instead of Ras overactivation as seen in human NF1 patients.[11] Despite the important insights such models provide, they appear as rather rough approximations of the human disease, leaving aside the subtleties of specific mutations found in patients of precise space- and time-controlled gene expression levels, etc. These limitations of the models are in part the basis of the limited use of *Drosophila* models of neurological diseases compared to rodent models, the latter often overcoming the above-mentioned limitations simply because the mouse genes and their regulatory elements are closer to those of humans.

I propose the systematic humanization of *Drosophila* genes encoding orthologs of neurology disease-related human genes as the means to establish and validate most disease-relevant *Drosophila* models of human neurological disorders. These models would then have a chance of taking over a significant portion of the animal model studies and rodent experimentation would be reduced. As an illustration of the 'success story' of such humanization for subsequent validation of a drug treatment, I discuss our recent modeling of pediatric *GNAO1* encephalopathies in *Drosophila*.[12–14]

2 | CASE STUDY: *GNAO1* ENCEPHALOPATHIES

GNAO1 encephalopathies affect infants and manifest as complex neurological disorders encompassing severe motor dysfunctions, epilepsy, developmental and intellectual delay, and occasional brain degeneration.[15,16] De novo, mostly single codon missense mutations in the *GNAO1* gene induce expression of neomorphic variants of the major neuronal G protein Gαo, resulting in abnormal functioning, dominantly affecting the wild-type protein's activities.[13,16,17] Insights into the aberrant Gαo functioning show the pathologic mutants as constitutively GTP-loaded yet unable to adopt the proper activated conformation, leading to aberrant interactions with cellular partners of the protein.[13] Attempts to model *GNAO1* encephalopathy in mice have been performed with limited success due to the neonatal lethality of the mutant animals.[18] This and the complexity of establishing the mouse models called for alternative models of the disease.

GNAO1 and its product Gαo are well-conserved between humans and *Drosophila*.[12,19] This prompted us to apply the humanization approach as a means to validate the usefulness of *Drosophila* as a potential host to model *GNAO1* encephalopathy. Using two-step CRISPR/Cas9-mediated engineering, we fully replaced *Drosophila* *Gαo* coding sequences with those leading to production of the human protein; at the same time, all the regulatory, non-coding parts of *Drosophila Gαo* remained intact in order to ensure the proper expression, in time, place, and levels, of the humanized protein.[12] Such an approach has been performed for systematic humanization of yeast genes,[20] but only episodically for the *Drosophila* genes.[21,22] Remarkably, humanized Gαo fully recapitulated multiple functions of the protein, including normal development, lifespan, behavior and memory formation,[12] while loss-of-function *Gαo* mutations led to embryonic lethality with numerous developmental defects.[19,23–25]

Inspired by this successful humanization of Gαo, we next moved to create a *Drosophila* model of pediatric *GNAO1* encephalopathy, choosing one of the hotspot *GNAO1* mutations, G203R affecting the amino acid conserved across species. Using CRISPR/Cas9, we introduced the pathologic mutation into one *Gαo* allele, keeping the other intact. The resulting heterozygous flies were viable yet recapitulated some of the disease manifestations, such as defective motor activity, reduced lifespan, and neurodegeneration.[13] What is more, we used this model of the human disease to validate a drug—zinc salts—that emerged from our in vitro screening of FDA-approved medications aimed at restoring proper GTP handling by the mutant Gαo. Already approved as a dietary supplement for multiple human disorders, dietary zinc added to the *Drosophila* food significantly rescued the aberrant motor activities and lifespan reduction in the *G203R/+* fruit flies.[13] Cumulatively, these findings led to application of dietary zinc to *GNAO1* encephalopathy patients, in off-label applications and in the preparation to multi-center clinical trials in several hospitals internationally.

3 | SOLUTION: *Drosophila* GENE HUMANIZATION AS A FIRST STEP TO NEUROLOGICAL DISEASE MODELING

Inspired by the example of modeling *GNAO1* encephalopathy in *Drosophila*, I suggest that this two-tiered approach—disease gene humanization followed by disease model establishment—should be upscaled to as many neurological diseases as possible. Success in the first tier of this approach will be a prerequisite to going on to the second, and serves as a filter for identifying the diseases that could and should be modeled in *Drosophila*. The adequacy of such models established using the CRISPR/Cas9 methodology, preferably based on the humanized genes (and primarily focusing on missense disease-causing mutations), will be guaranteed by the prior successful humanization. This will give such models the credibility that will ensure and promote their wide usage among fundamental and applied researchers world-wide. I anticipate that this will contribute significantly to a reduction in the use of rodents, which will be replaced by the relevant *Drosophila* models of fundamental and translational neurology-related experimentation, and will also contribute significantly to our understanding of the details of the molecular etiology of the diseases and to developing treatments against them.

The approach I am advocating here goes far beyond that used in a recent screening of de novo variants of autism in *Drosophila*.[26] In this important work, a collection of transgenic lines was created, in which *Drosophila* homologs of human autism genes were knocked-out and replaced with a Gal4 driver. Crossing the resulting strain with a line transgenic for a UAS-driven human version of the protein (or its pathogenic variant) then permitted analysis of the eventual phenotypes emerging from the variants of human autism-related protein. Although a step forward in current approaches to model neurological diseases in *Drosophila*, this approach[26] has clear limitations: regulatory sequences (and neighboring / intervening genes) of the fruit fly genes are disturbed by the Gal4 insertions; Gal4-UAS-driven expression of the human variants inevitably amplifies the protein levels beyond those of the parental *Drosophila* counterpart; insertion of the UAS construct to a site in the genome distinct from that of the parental *Drosophila* gene imposes further artificial restrains on the expression time, place, and levels. The approach I am proposing, in contrast, aims at humanization of the disease-related *Drosophila* genes while maintaining their natural expression and its regulation and fully preserving the neighboring sequences.

4 | NEUROLOGICAL DISEASE GENE CANDIDATES FOR HUMANIZATION IN *Drosophila*

Which neurological diseases, associated to which genes should be subjected to the *Drosophila* humanization approach? The candidates to start with are those where sequence similarities between the human disease gene and its *Drosophila* ortholog are high. To identify such candidates, proteins encoded by the human neurological disease-related genes[27] have been systematically aligned with their *Drosophila* orthologs (Figures S1), producing the list of genes to be systematically humanized in *Drosophila* (Table 1). Table 1 further lists the neurological diseases caused by mutations in these genes. Some of these neurological diseases are rather rare, such as lissencephaly, which occurs with a prevalence of 12–40 cases per million births.[28] Others, in contrast, are very common, such as amyotrophic lateral sclerosis (ALS) associated with mutations in *SOD1* (ca. 20% familial ALS and ca. 2% sporadic ALS),[29] neurofibromatosis type 1 with a prevalence of about 1/3000,[30] Alzheimer's disease associated with *PSEN1* mutations, or the Niemann-Pick disease associated with mutations in *NPC1*. Each of the genes implicated in these diseases in humans has a strong ortholog in *Drosophila* (Table 1 and Figures S1).

I now briefly discuss some representatives of the neurological diseases and their underlying genetic mutations from Table 1, focusing on the molecular functions of the proteins affected, availability of the rodent models, and the prospects of their respective gene humanization in *Drosophila*.

Miller-Dieker syndrome (MDS) and many cases of a related disease designated as isolated lissencephaly sequence (ILS) display a severe neuronal migration disorder resulting in a smooth vertebral surface, seizures and mental retardation. One of the main culprits in these lissencephalies is *LIS1* (*PAFAH1B1*), which encodes an atypical microtubule associated and dynein-regulating protein LIS1, also known as platelet-activating factor acetylhydrolase IB subunit beta; deletions encompassing *LIS1* underlie the disease, revealing the haploinsufficiency of this gene.[31] In mice heterozygous for loss-of-function in *LIS1*, disorganization in several brain areas, resulting from delayed neuronal migration, led to impaired learning and motor behavior with occasional seizures,[32,33] validating this rodent model of the human disease. Interestingly, a number of C-terminal truncating and missense mutations in *LIS1* have also been identified in lissencephalies.[34] In *Drosophila*, loss-of-function mutations including point and truncating mutations in *Lis-1* have been found to aberrate germline cell division and oocyte differentiation,[35] nuclear migration,[36] and neuroblast proliferation and dendrite formation.[37] However, *Lis-1* is not haploinsufficient in the fruit fly and mutations in both alleles are required to produce phenotypes.[35–37] The *Lis-1* gene in *Drosophila* has a relatively simple structure with 5 protein-coding exons interspersed with short introns, without any overlapping gene (flybase.org/reports/FBgn0015754),[35] encoding a 411 amino acids-long protein (46.5 kDa) with 70% identity and 87% similarity with the human LIS1 protein (Figure S1A). I suggest humanization of the *Drosophila Lis-1* gene (its protein-coding exon cluster) using the human protein-coding sequences and maintaining the flanking and intronic regulatory *Drosophila* sequences with CRISPR/Cas9. Both wild-type and disease-causing point/truncating mutation-harboring human sequences should be introduced. The ability of the human coding-sequences replacing the endogenous *Drosophila* sequences to recapitulate the normal development and functioning of the fruit fly will be an important step forward. It will further be of prime importance for modeling lissencephaly in *Drosophila* if the humanized Lis-1 proves to be haploinsufficient; the possibility of some of the disease point mutations emerging as dominant should also be investigated.

Neurofibromatosis type 1 (NF1) is a complex disorder encompassing peripheral nervous system tumors such as cutaneous and plexiform neurofibromas and malignant peripheral nerve sheath tumors. Other features are traits include pigmentary lesions, optic gliomas, and skeletal lesions. In addition, affected individuals also develop learning disabilities and behavioral problems such as attention-deficit/ hyperactivity disorder. NF1 is caused by inherited or de novo mutations in the *NF1* tumor suppressor gene[38] encoding neurofibromin—a multidomain protein of ca. 2800 amino acids, 55.5% identical and 69.8% similar to the *Drosophila* neurofibromin 1 (Table 1, Figure S2). A key region in neurofibromin is the RasGAP domain, mediating GTP hydrolysis and deactivation in small GTPases of the Ras subfamily; loss of this activity is believed to underlie the oncogenic manifestations in NF1.[39] Although many cases of NF1 are caused by heterozygous loss-of-function mutations such as deletions, numerous heterozygous point mutations have also been identified in patients. Importantly, at least some of these point mutations result

TABLE 1 Examples of neurological disease genes with strong orthologs in *Drosophila*.

Neurological disease gene	Disease	Encoded protein	*Drosophila* ortholog	*Drosophila* protein	% identity	% similarity
PAFAH1B1 (LIS1)	Miller-Dieker lissencephaly	Platelet-activating factor acetylhydrolase IB subunit beta (LIS1)	*Lis-1*	Lissencephaly-1 homolog	70.0	87.0
PAH	Phenylketonuria	Phenylalanine-4-hydroxylase	*Hn (pah, Tph)*	Protein henna	62.3	77.3
SOD1	Amyotrophic lateral sclerosis	Superoxide dismutase [Cu-Zn]	*Sod1*	Superoxide dismutase [Cu-Zn]	61.6	74.2
NF1	Neurofibromatosis, type 1	Neurofibromin	*Nf1*	Neurofibromin 1, isoform B	55.5	69.8
PSEN1	Alzheimer disease	Presenilin-1	*Psn*	Presenilin homolog	51.5	63.0
NF2	Neurofibromatosis, type 2	Merlin	*Mer (EMR2)*	Moesin/ezrin/radixin homolog 2	47.8	65.0
NPC1	Niemann-Pick disease	NPC intracellular cholesterol transporter 1	*Npc1a*	Niemann-Pick type C-1a, isoform A	46.4	62.8
PRKN	Juvenile onset Parkinson's disease	E3 ubiquitin-protein ligase parkin	*Park*	E3 ubiquitin-protein ligase parkin	42.8	58.7
UBE3A	Angelman syndrome	Ubiquitin-protein ligase E3A	*Ube3a*	LD21888p	41.2	59.2
FXN	Friedreich's ataxia	Frataxin, mitochondrial	*fh*	Frataxin homolog, mitochondrial	40.2	55.5
ATP7B	Wilson's disease	Copper-transporting ATPase 2	*ATP7*	P-type Cu(+) transporter	40.1	53.5
PEX5 (PXR1)	Zellweger syndrome	Peroxisomal targeting signal 1 receptor	*Pex5*	EG:63B12.5 protein	37.1	52.1
CAPN3	Limb girdle muscular dystrophy 2A	Calpain-3	*CalpB*	Calpain-B	36.1	50.4
BEST1 (VMD2)	Best macular dystrophy	Bestrophin-1	*Best1*	Bestrophin homolog	35.7	49.0
GBA1	Gaucher disease	Lysosomal acid glucosylceramidase (glucocerebrosidase)	*Gba1a*	Glucosylceramidase	35.4	53.1
FMR1	Fragile X syndrome	Fragile X messenger ribonucleoprotein 1	*Fmr1*	Fmr1, isoform I	35.3	48.4
LMNA (LMN1)	EmeryDreifuss muscular dystrophy	Prelamin-A/C	*Lam*	Lamin	35.0	52.2
TSC2	Tuberous sclerosis	Tuberin	*Gig*	Gigas, isoform A	34.8	52.0
DMD	Duchenne muscular dystrophy	Dystrophin (Dystrophin-4)	*Dys (det)*	Dystrophin, isoforms A/C/F/G/H	33.2	50.6
HEXA	Tay-Sachs disease	Beta-hexosaminidase subunit alpha	*Hexo2*	Beta-N-acetylhexosaminidase	32.7	45.7
OCRL	Lowe oculocerebrorenal syndrome	Inositol polyphosphate 5-phosphatase OCRL	*Ocrl*	Phosphoinositide 5-phosphatase	31.4	47.0
ABCA1	Tangier disease	Phospholipid-transporting ATPase ABCA1	*Abca3*	ATP binding cassette subfamily A, isoform D	30.4	44.7

Note: % identity and similarity counted with the VectorBuilder sequence alignment tool.

in more severe disease manifestations than loss-of-function mutations, hinting at possible dominant mechanisms.[40] Mouse models have been established, wherein heterozygous loss-of-function mutations in *NF1* recapitulate some of the patients' clinical manifestations, including predisposition to tumor development and learning and memory impairments.[41,42] In *Drosophila*, heterozygous mutations in *Nf1* do not produce any detectable phenotypes, while homozygous nulls are small in size and display perturbed neuronal signaling and reduced life span.[11,43,44] *Nf1* in *Drosophila* contains 16 protein-coding exons interspersed with short introns, without any overlapping gene (flybase.org/reports/FBgn0015269). I suggest that CRISPR/Cas9-based humanization of the *Drosophila Nf1* gene (its protein-coding exon cluster) should be performed using the human protein-coding sequences, maintaining the non-protein coding *Drosophila* sequences, flanking and intronic. It will be crucial for the understanding of neurofibromin function and evolution if the human protein that replaces the endogenous fruit fly ortholog but maintains the *Drosophila*-specific expression is able to mediate normal fly development and behavior. Moreover, such humanization will permit establishment of valid *Drosophila* models of NF1 upon introduction into the humanized sequence of the missense mutations that cause more severe disease manifestations in patients, especially if such mutations produce phenotypes in *Drosophila* in the heterozygous setting.

Another example of a neurological disease gene from Table 1 is *PRKN*, which is mutated in an autosomal recessive manner in juvenile parkinsonism. It encodes a 465-amino acid-long E3 ubiquitin ligase, and loss of this enzyme leads to defective control over levels of a number of proteins that ultimately leads to degeneration of dopaminergic neurons in the substantia nigra and early-onset of Parkinson's disease.[45] A large set of mutations including deletions, frameshifts, and missense mutations have been observed in patients; curiously, gain-of-function has been attributed to some of the missense mutations.[46,47] Mouse modeling of juvenile parkinsonism has been attempted by creating knockout animals, but the homozygous animals did not reveal any loss of dopaminergic neurons despite having moderate behavioral/motor deficits.[48] Interestingly, homozygous loss-of-function mutations in *Drosophila* provided closer similarity to human patients, with loss of dopaminergic neurons and strong motor dysfunction, alleviated by L-DOPA administration.[49] The *park* gene in *Drosophila* is compact, with six protein-coding exons interspersed with short introns, without any overlapping genes (flybase.org/reports/FBgn0041100), encoding the protein, which is ca. 43% identical and ca. 59% similar to its human ortholog (Table 1 and Figure S3). As in the examples above, humanization of *Drosophila park* should involve CRISPR/Cas9-based substitution of the protein-coding exons with the humanized sequence, sparing the non-coding flanking and intronic sequences to ensure proper expression and regulation of the gene. Such humanization will provide insights into the function and conservation of parkin, and will also permit introduction of hotspot disease-causing missense mutations[46] into the humanized sequence, advancing our understanding of the disease etiology in the validated *Drosophila* model of the disease.

5 | CONCLUSIONS

The examples above illustrate that, across different protein types, humanization of the neurological disease-causing proteins in *Drosophila* can be a powerful tool for validating the applicability of the fruit fly as a model of the diseases. This approach further promises to provide insights into the molecular etiology of the diseases, especially in relation to missense mutations. Drug discovery opportunities are also expected to result from this humanization strategy. I thus call for the initiation of a massive project dedicated to the systematic humanization of *Drosophila* focusing on the neurology disease-associated genes, following recent examples.[12,13] I suggest that such a project would be best performed as a single large-scale effort in a laboratory with expertise in CRISPR/Cas9-based mutagenesis and the genetics of *Drosophila*, humanizing the genes from Table 1 and characterizing the resultant lines. These lines will be openly shared with the scientific community, initially through lab-to-lab sharing and ideally later by depositing the whole collection in one of the public stock centers, such as the Bloomington Drosophila Stock Center (bdsc.indiana.edu/). This project will create a powerful toolset to advance understanding of the molecular etiology of neurological diseases, along with models amenable to drug discovery and development, and will contribute significantly to a reduction in the use of experimental rodents by replacing them with adequate *Drosophila* models.

ACKNOWLEDGMENTS

I thank Pierre Cosson for critically reading and discussing the manuscript, and Mikhail Savitsky for creating the graphical abstract.

FUNDING INFORMATION

This work was supported by Swiss National Science Foundation, grant #31003A_175658 to VLK.

CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

ETHICS STATEMENT

None.

DATA AVAILABILITY STATEMENT

The research data described in the paper is presented in full in the text, figures, and table of the paper.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Katanaev VL. Humanization for neurological disease modeling: A roadmap to increase the potential of *Drosophila* model systems. *Anim Models Exp Med*. 2023;6:230-236. doi:10.1002/ame2.12322