Genetic models of *C9orf72*: What is toxic?

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Abstract

A hexanucleotide repeat expansion in the gene *C9orf72* is the most common genetic cause of both amyotrophic lateral sclerosis and frontotemporal dementia. Pathogenesis may occur either due to loss of function of the *C9orf72* gene, or a toxic gain of function, via the production of repetitive sense and antisense RNA and/or repetitive dipeptide repeat proteins. Recently, mouse knockouts have suggested that a loss of function of *C9orf72* alone is insufficient to lead to neurodegeneration, whilst overexpression of hexanucleotide DNA is sufficient in a wide range of model systems. Additionally, models have now been created to attempt to study the effects of repetitive RNA and dipeptide proteins in isolation and thus determine their relevance to disease.

Introduction

A non-coding hexanucleotide repeat expansion in *C9orf72* is the most common genetic cause of both frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS)[1,2]. The mutation is a (GGGGCC)$_n$ expansion with unaffected individuals typically carrying fewer than 30 repeats, while patients harbour several hundreds to thousands[1–4]. Affected individuals present clinically with either ALS, FTD, or both [1,2,5] and display TDP-43 pathology[1,6].

Three mechanisms have been proposed to explain how *C9orf72* hexanucleotide expansions lead to ALS/FTD (C9ALS/FTD), either by loss-of-function or by two different gain-of-function mechanisms (Figure 1A-C):

(1) The expansion leads to reduced transcription of *C9orf72*, leading to haploinsufficiency (Figure 1A).

(2) Repeat-containing sense and antisense RNA transcripts accumulate and sequester RNA binding proteins (Figure 1B).

(3) Sense and antisense RNA are translated by repeat-associated, non-ATG initiated (RAN) translation into five, potentially toxic, repetitive dipeptide proteins (DPRs) (Figure 1C).

We will critically review newly developed cellular and animal C9FTD/ALS models and discuss the insight they provide into the role of each proposed mechanism in disease pathogenesis.

Loss of function

C9orf72 has three splice variants that either harbour the expansion in the first intron of the gene (variants 1 and 3) or the promoter (variant 2)[1]. Variant 1 comprises exons 1-5 and encodes a short protein isoform (222 amino acids: C9ORF72-S) while variants 2 and 3 encode the long isoform (481 amino acids: C9ORF72-L)[1,7].

Analysis of post mortem C9ALS/FTD brains showed reduced *C9orf72* transcript [1,8–13] and reduced protein[7,11,14]. This may be a consequence of epigenetic silencing[8,10,15,16] or abortive transcription[17]. Reduced *C9orf72* levels in patients led to the suggestion that loss of normal *C9orf72* function leads to pathogenesis. However, only a single sporadic ALS patient has ever been found to carry a mutation potentially capable of causing a heterozygous loss of function, and it is unclear if this mutation is causative of the disease state[18,19]. Additionally, a patient homozygous for the repeat expansion had a similar clinical phenotype to heterozygote patients, rather than a much more severe phenotype typical of homozygous loss of function mutations[12]. Therefore the genetic evidence currently supports a gain of function rather than a loss of function mechanism as the primary cause of disease [20]. However, loss of function models are providing evidence in favour of an important role for *C9orf72* in cellular functions relevant to neuronal function, which suggests loss of function could contribute to the disease process.

C9orf72 is homologous to members of the DENN (differentially expressed in normal and neoplastic cells) domain containing protein family, and is predicted to be involved in membrane trafficking as a Rab guanine exchange factor[21]. C9ORF72-L forms a complex with SMCR8 and WDR41[22–27] and is involved in the induction of autophagy[22,23,25,28,29], whilst C9ORF72-S is localised at the nuclear membrane in patient brain and has been suggested to be involved in nucleocytoplasmic transport[7]. Work in cell lines or primary rodent neuronal cultures has demonstrated that knockdown of *C9orf72* is sufficient to induce P62-positive puncta[23,28], consistent with autophagic dysfunction, and one study has observed the mislocalisation of phosphorylated TDP-43 after *C9orf72* knockdown[23].

Several animal models have been produced in which the expression of genes orthologous to *C9orf72* are reduced (Table 1, Figure 1D). Knockout of the *C. elegans* ortholog of *C9orf72* (ALFA-1) led to progressive age-related paralysis[30]. Injection of antisense morpholino oligonucleotides during zebrafish development led to altered axonal branching in larval motor neurons and an associated motor phenotype[9]. Consistent with this finding, it was recently shown that *C9orf72* regulates actin dynamics and axon growth in cultured motor neurons[14].

Several groups have tested the effect of reduced expression of the mouse ortholog of *C9orf72* (3110043O21Rik), either in a tissue-specific or ubiquitous manner (Table 1). The results of these studies are strikingly concordant; no overt neurodegeneration occurs in response to partial or complete loss of *C9orf72* function. Furthermore, no mislocalisation of TDP-43 is observed[31–33]. These findings clearly suggest that loss of *C9orf72* function is not sufficient to cause disease. Interestingly, however, whilst neuron-specific knockdown or knockout had no pathogenic effects[31,34], whole-genome knockout resulted in immune system phenotypes, including splenomegaly (enlarged spleen) and lymphandenomegaly (enlarged lymph nodes) [25,33,35–37]. These findings are particularly interesting given the increasing evidence supporting a role for the immune system in a wide range of neurodegenerative diseases[38].

Gain of RNA and Protein Function

Gain of Function Pathologies

A typical feature of many repeat expansion diseases is the formation of RNA foci: aggregates of RNA which are capable of sequestering RNA-binding proteins [39]. In C9ALS/FTD, repeat-expanded RNA is transcribed in both sense and antisense directions and forms both nuclear, and rarer, cytoplasmic sense and antisense RNA foci across multiple patient brain regions, and in patient derived cell lines (Figure 1B) [1,34,40–48]. Several studies have identified RNA-binding proteins (RBPs) sequestered by sense and antisense transcripts. Therefore reduced availability of these RBPs may play a role in the disease state (reviewed in [49]).

In addition to the formation of RNA foci, sense and antisense expansion-containing RNA is also RAN translated in all three reading frames, producing five, potentially toxic dipeptide-repeat proteins (DPRs)[43,50,51] (Figure 1C). DPRs form p62-positive, TDP43-negative inclusions, abundant in the neocortex, hippocampus and cerebellum[50–52]. Typically, poly-GA inclusions are the most abundant, followed by poly-GP and poly-GR, whilst poly-AP and poly-PR inclusions are rare [47,53,54]. Pathological studies have examined DPR inclusions in C9FTD, C9ALS or C9FTD/ALS patients, and have not found consistent correlations between RAN protein pathology and clinical phenotype, TDP-43 pathology or neurodegeneration [52,53,55]. However, a recent study found that levels of soluble poly-GP in the cerebellum correlate positively with cognitive impairment [56].

Pure GGGGCC repeat models

In order to determine whether a gain of function is sufficient to induce a disease phenotype, several groups have overexpressed GGGGCC repeats and demonstrated deleterious phenotypes in cell culture [45,57–59], *C.elegans* [60] , Zebrafish[45], and *Drosophila*[58,61–63] models, providing evidence that *C9orf72* mutation associated toxic gain of function is sufficient to cause disease (Figure 1E).

Recently, several mouse models of *C9orf72* expansions have been produced, either using overexpression of hexanucleotide repeats [64], or integration of human bacterial artificial chromosomes (BACs) containing expanded hexanucleotide DNA[35,37,66,67] (Table 2). Whilst all studies demonstrated that expression of

repeat-expanded RNA led to foci formation, and resulted in at least some DPR species, the resultant phenotypes of the various mouse models were markedly different.

Overexpression of 66 GGGGCC repeats using adeno-associated viruses (AAVs) led to accumulation of RNA foci, DPR and TDP-43 pathology as well as neuronal loss with cognitive and motor dysfunction[64]. Generation of BAC mice led variously to: no cognitive or motor abnormalities[65,66], hippocampus-related cognitive dysfunction[37] or either acute or progressive phenotypes marked by behavioural and motor abnormalities and reduced survival[67]. The discrepancies between these outcomes seem unlikely to be due to differences in the amount of 5' or 3' *C9orf72* flanking sequence present in each BAC, as the BACs from phenotypic and non-phenotypic mice overlapped (Table 2). It is notable that the different models were produced in different genetic backgrounds (Table 2), which can strongly influence neurodegenerative phenotypes in mice [68]. Additionally, toxicity may be dependent on level of expression. For example, Chew et al. (2015) [64] and Liu et al. (2016) [67] found that strong overexpression of relatively short repeat lengths to be toxic, whilst Jiang et al. (2016) [37] found that foci formation and DPR accumulation were positively correlated with repeat-expansion-containing RNA expression levels. It is thus possible that the required level of putatively toxic RNA or DPR species was not met in non-phenotypic mice.

Despite these differences, the fact that expression of hexanucleotide RNA can lead to behavioural and motor abnormalities underpinned by neurodegeneration, especially in a BAC context where expression is driven by the endogenous *C9orf72* promoter, strongly suggests that a gain of function alone is sufficient to drive pathogenesis. It is notable that phenotypic mice either displayed TDP-43 inclusions[64,67] or increased TDP-43 phosphorylation[37], providing further evidence that neurodegeneration is driven primarily through a toxic gain of function leading to TDP-43 pathology.

RNA or Protein?

Whilst pure GGGGCC repeat expression models provide evidence of a toxic gain of function, they cannot distinguish whether toxicity is primarily mediated by RNA or DPRs, which has led to considerable debate in the field.

RNA-only models

In order to study the potential role of RNA toxicity in isolation, it is necessary to design models where GGGGCC repeat containing RNA is expressed whilst RAN translation is suppressed. GGGGCC repeat RNA (36 and 103 repeats) is toxic to *Drosophila* neurons when overexpressed. Conversely, when the repeats are interrupted by regularly interspersed stop codons in every reading frame RAN translation and toxic phenotypes are completely prevented, even when up to ~288 interrupted repeats are expressed[61,69]. This suggests that DPRs are the major contributor to toxicity in *Drosophila* models of *C9orf72*. In concordance with this conclusion, overexpression of sense hexanucleotide RNA from the intron of a transgene in *Drosophila* is sufficient for the formation of numerous RNA foci but does not cause toxicity, because efficient splicing of intronic repeat RNA prevents its nuclear export and RAN translation[70] (Figure 1F).

Interestingly, in a BAC mouse model, the presence of multiple copies of short repeat lengths (29-36 repeats) produced only DPRs and not nuclear RNA foci, but TDP-43 pathology and neurodegenerative phenotypes were still observed[67](Table 2). Together these results suggest that RNA foci are dispensable for the induction of disease pathogenesis in *Drosophila* and mice. However, it is difficult to rule out a possible toxic effect of RNA that does not form foci, or cytoplasmic RNA, a prospect that has recently been raised in iPSC-derived neurons[63].

On the other hand, a different *Drosophila* model carrying an inducible construct capable of expression of 30 hexanucleotide repeats interrupted by a 6-base pair restriction endonuclease cut site[58] does not produce detectable DPRs when expressed in photoreceptor neurons or pan-neuronally despite showing degenerative phenotypes, suggesting that toxicity in this model is primarily driven by RNA[71]. It should be noted however, that poly-GR becomes detectable upon strong overexpression in these flies, suggesting that there may be poly-GR present at

undetectable levels, which may contribute to the observed phenotypes in lower expressing flies. Additionally, it has been shown that expression of 42 GGGGCC repeats from the intron of a transgene is toxic to mouse primary cortical and motor neurons, with the development of abundant RNA foci but no detectable DPR accumulation[59].

These results suggest that the potential toxicity of expanded RNA may depend on subcellular localisation and the model system employed. Additionally, no study has attempted to assess the effect of overexpression of antisense RNA in isolation. Thus, further characterisation of existing models as well as development of antisense models will be required to fully assess the contribution of RNA toxicity in C9ALS/FTD.

Protein-only models

Protein-only models include exogenous application of recombinant DPR peptides and the use of codon degeneracy (individual amino acids can be encoded by more than one triplet codon) to generate transgenic models that overexpress individual DPRs in an ATG-driven manner, allowing comparison of their relative effects (Figure 1G, Table 3). Although DPRs may be present at higher levels than those typically observed in patient tissue, there is a broad consensus across multiple model systems that arginine-rich DPRs (poly-GR and Poly-PR) are highly toxic (Table 3), with other dipeptide proteins, most notably poly-GA, being identified as toxic in some model systems [61,72–75].

In order to determine mechanisms of DPR toxicity, several groups have attempted to identify arginine-DPR interacting proteins using immunoprecipitation of DPRs from cell lines with subsequent mass-spectrometric identification of interacting proteins[76–80]. Recently, two studies have demonstrated that arginine-rich DPR interactors are enriched in low complexity regions (LCRs), typically found in protein constituents of membrane-less organelles such as stress granules, nuclear speckles and nucleoli[79,80]. The exact mechanism(s) by which the arginine-rich DPRs are toxic to neurons are still not fully resolved, however translational repression[77,80], mitochondrial dysfunction[78], nucleolar dysfunction[76,81], and disruption of nucleocytoplasmic transport have all been implicated[62,71,82,83].

It should be noted that although poly-GA is seemingly less toxic than the arginine rich DPRs, it has been shown to exert mild toxicity when expressed in *Drosophila* neurons[61] and in cell culture, including primary rodent neurons[72–75]. Recently it was demonstrated that AAV-mediated overexpression of poly-GA dipeptides in mice led to neurodegeneration and, cognitive and motor dysfunction[74]. However, only rare TDP-43 inclusions were observed, indicating that accumulation of poly-GA protein alone may be insufficient to lead to the full disease state[74]. Future studies will be required to determine whether arginine-rich DPR overexpression is sufficient to induce ALS/FTD associated phenotypes in mice.

Synergistic Mechanisms

Although researchers have aimed to isolate different possible mechanisms of toxicity in order to elucidate their potential role in disease, it is possible that individual mechanisms act synergistically to induce the disease state.

Whilst loss of function of *C9orf72* alone is unlikely to be sufficient to induce disease, cell culture studies suggest that reduced expression of *C9orf72* leads to disruption of autophagy[22,23,28,29] and autophagic dysfunction has been strongly linked to neurodegenerative disease [84]. Therefore the potential role of *C9orf72* loss of function as a disease modifier should be thoroughly explored. Recently it was demonstrated that overexpression of *C9orf72* reduces the aggregation of TDP-43 in cell culture and a small decrease in *C9orf72* expression exacerbated expanded ataxin-2 pathology in mouse neurons and zebrafish larvae[23]. The genetic models described above, such as knockout mice and mice transgenic for hexanucleotide expansions can now be combined to ascertain the degree to which *C9orf72* loss of function may affect disease phenotypes.

Arginine-rich DPRs are toxic in multiple systems whilst poly-GA has also been shown to be toxic to mouse neurons. Interestingly, poly-GA has been demonstrated to recruit poly-GR into inclusions when co-expressed in human cells [85] and in *Drosophila*, resulting in a lessening of poly-GR toxicity [86]. Whether interaction between the individual DPRs acts to enhance or suppress their toxicity is thus a point of interest. Additionally, frameshifting during the translation of repetitive proteins has been described in other repeat expansion disorders[87]. Whether this occurs in

C9orf72 mutation carriers is currently unknown, but could potentially lead to the production of peptides imbued with the qualities of more than one DPR species.

Conclusions

Recent studies of the mechanism(s) of pathogenesis in C9ALS/FTD have focused on attempting to separate potential pathologies in model systems to study them further. Although the C9ORF72-L protein has been implicated in the induction of autophagy, a number of mouse loss of function models have failed to develop ALS/FTD associated phenotypes (Table 1). Expression of expanded GGGGCC repeat DNA is extremely toxic in a number of model systems. Recent advances include the expression of GGGGCC repeat DNA in mice, which under some circumstances display motor dysfunction and behavioural abnormalities with TDP-43 pathology (Table 2). Despite the potential shortcomings of the model systems in question, for example the incomplete homology of disrupted orthologous genes (see Table 1), or the strong overexpression of relatively short repeat lengths employed in many models, in combination these results strongly indicate that expression of expanded GGGGCC repeats alone are sufficient to cause C9ALS/FTD.

Although it seems likely that the *C9orf72* repeat expansions cause toxicity through a gain of function it is not clear whether this is primarily mediated by expanded RNA or DPR proteins. Several groups have attempted to separate RNA expression from DPR production, with clear evidence, albeit in *Drosophila* models, indicating that repeat RNA without the expression of dipeptide proteins may not be toxic. Others have studied the relative toxicity of different DPR species by overexpressing recodonised protein constructs (Table 3) and overexpressed arginine-containing DPRs have been repeatedly independently identified as being toxic. However, overexpression of poly-GA is sufficient to induce neurodegeneration in mice, leaving the relative contribution of different dipeptides to the toxicity demonstrated in mouse models currently unresolved. It will be important to develop models with physiological expression levels of the DPRs in order to accurately tease out the contribution of each DPR to neuronal dysfunction. It is also likely that the different potential mechanisms of toxicity interact in bringing about the disease state. Thus, although individual DPRs and hexanucleotide containing RNA have the potential to be toxic, the combination of individual, physiological genetic models will be crucial in understanding how these pathologies interact and ultimately lead to the full disease state.
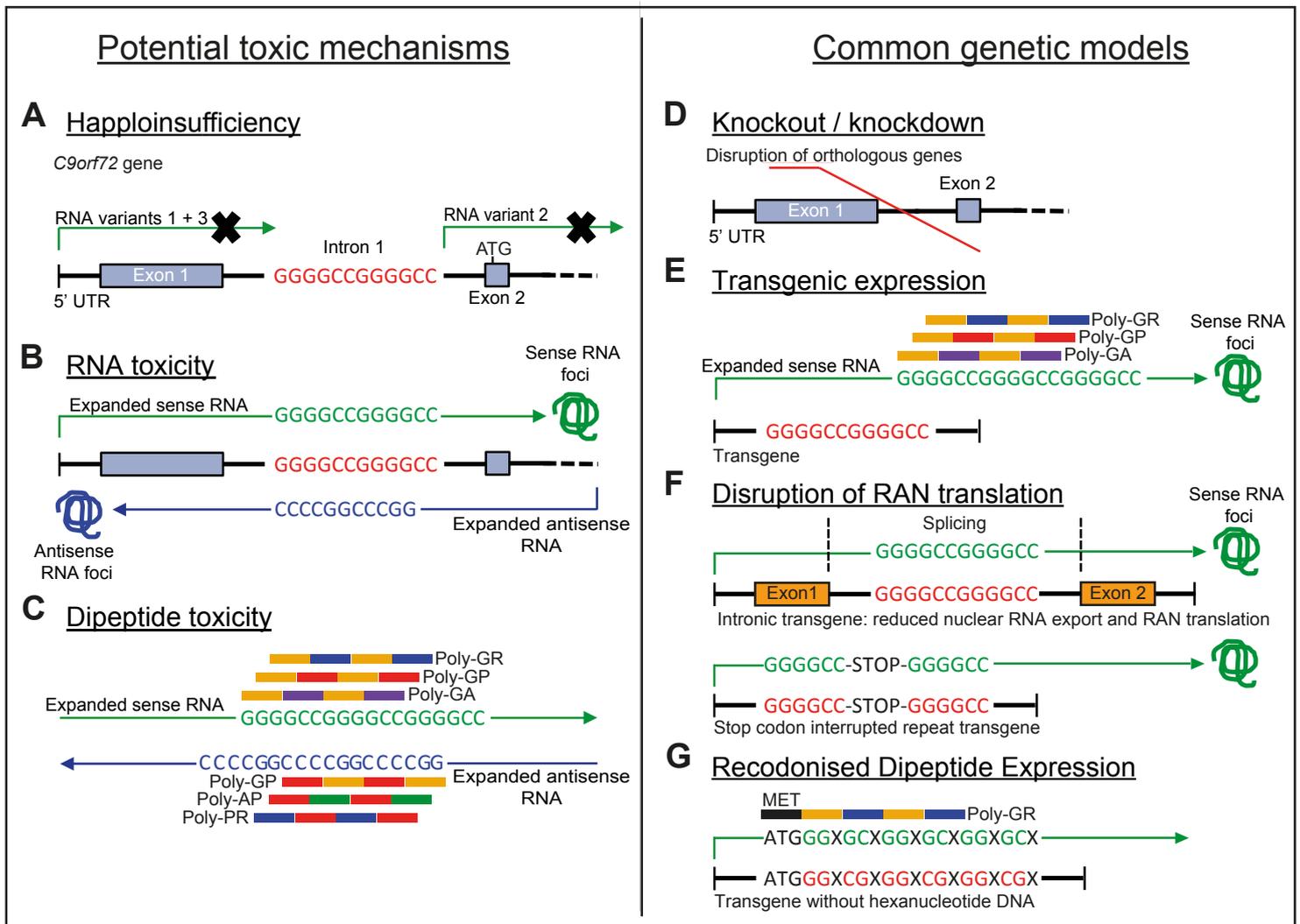
Figure 1: Potential mechanisms of *C9orf72* hexanucleotide expansion toxicity and commonly employed genetic models. Three potential mechanisms by which the C9orf72 associated hexanucleotide expansioncauses toxicity. **A)** Epigenetic silencing associated with the repeat expansion leads to reduced *C9orf72* expression and thus happloinsufficiency. **B)** The hexanucleotide expanded DNA is transcribed in both sense and antisense directions. These expanded RNAs form typically, intranuclear aggregates known as RNA foci, which have been proposed to sequester RNA binding proteins leading to toxicity. **C)** Repeat expanded RNAs can undergo repeat-associated-non-ATG-initiated (RAN) translation producing repetitive peptides from all reading frames without the requirement of an ATG start codon[57]. RAN translation of both sense and antisense RNA has been demonstrated to occur in C9ALS/FTD patients, leading to the production of five potentially toxic dipeptide repeat proteins (DPRs), these are: poly-glycine-alanine (poly-GA), poly-glycine-proline (poly-GP), poly-glycine-arginine (poly-GR), poly-alanine-proline (poly-AP), and poly-proline-arginine (poly-PR). **D)** Loss of function of *C9orf72* or its orthologous genes in other species can be used to study whether a complete or partial loss of *C9orf72* in isolation is sufficient to induce a disease phenotype (see Table 1) **E)** Transgenic overexpression of GGGGCC repeats, either on their own or as part of a patient derived bacterial artificial chromosome (BAC), model both RNA and DPR toxicity, an approach that has recently been applied to generate mouse models of the disease (Table 2). **F)** Two approaches have been used to suppress RAN translation and study the effect of hexanucleotide repeat expression in isolation, either expression of relatively short stretches of GGGGCC repeats from within a strongly constitutively spliced intronic region[59,70] or by regularly interrupting the repeat sequence with stop codons[61]. **G)** Codon degeneracy allows for the engineering of "recodonised" constructs that allow ATG-initiated expression of individual dipeptide repeat proteins without an underlying GGGGCC repeat sequence (Table 3).

Model Organism	Orthologue (%protein identity)	Intervention	Phenotype	Study(s)
C.elegans	ALFA-1 (23.6%)	Knockout/RNAi silencing	Enhanced age-related paralysis phenotype. GABAergic neuronal dysfunction	Therrien et al. (2013) [30]
D.rario	zC9orf72 (76.1%)	Antisense morpholino injection into fertilized eggs	Shortened motor neuron axons, deficits in evoked and spontaneous swimming in larvae	Ciura et al. (2013) [9]
M.musculus	3110043O21Rik (98.1%)	Intraventricular injection of antisense oligonucleotides against C9orf72	No behavioural or motor differences. No evidence of TDP-43 pathology.	Lagier-tourenne et al. (2013) [34]
		Cre-lox mediated brain specific excision of exons 4-5	In both heteroyzgous and homozygous animals: No alteration in lifespan or grip strength. No evidence of TDP-43 pathology or neurodegeneration.	Koppers et al. (2015) [31]
		Transgenic mice with replacement of exons 2-11 with LacZ construct	Reduced motor function in homozygous knockouts. Evidence of immune dysregulation. No overt neurodegeneration.	Atanasio et al. (2016) [37]
		Transgenic mice with replacement of exons 2-6 with LacZ construct	Reduced lifespan in both heterozygote and homozygote knockouts. Evidence of immune dysregulation. No evidence of neurodegeneration.	Suzuki et al. (2013) [87]; Burberry et al. (2016) [35]
		CRISPR-cas9 targeted disruption of exon 4	Reduced survival in mosaic heterozygous and compound heterozygous mutants. Evidence of immune dysfunction.	Burberry et al. (2016) [35]
		Transgenic mice with replacement of exons 2-6 with LacZ construct	Reduced lifespan in homozygous knockouts. Some mild beahvioural and motor dysfunction. Evidence of immune dysregulation. No overt neurodegeneration.	Jiang et al. (2016) [36]
		Transgenic mice with replacement of exons 2-6 with LacZ construct	No behavioral or motor differences. Evidence of immune dysfunction. No evidence of TDP-43 pathology or neurodegeneration.	O'Rourke et al. (2016) [33]
		Zinc finger nuclease mediated removal of exon 2 start codon		O'Rourke et al. (2016) [33]
		Cre-lox mediated whole-genome specific excision of exons 4-5	Homozygous knockouts display reduced survival. No evidence of motor dysfunction. No evidence of TDP-43 pathology or neurodegeneration. Evidence of immune deregulation.	Koppers et al. (2015) [31]; Sudria Lopez et al. (2016) [32]
		CRISPR-cas9 mediated frameshift mutation in exon 2 causing premature stop codon affecting variants 1 and 3.	Evidence of immune dysregulation	Sullivan et al. (2016) [25]

Table 1: Comparison of loss of animal studies examining phenotypic effects of loss of function of C9orf72 orthologs.

Type of Transgenic	Transgene Information	Repeat Length	Genetic Background	Phenotype	Foci Pathology		Dipeptide Pathology					TDP-43 Inclusions	Neurodegeneration	Study
					Sense	Antisense	GP	GA	GR	PA	PR			
AAV based overexpression with CMV promoter.	CMV promoter. Intracerebroventricular injection at P0. Repeats cloned with 119bp 5' flanking region and 100bp 3' flanking region of the *C9orf72* gene	2 repeats	C57BL/6J	Male mice examined. 66 repeat expressing mice demonstrate behavioural and motor abnormalities.	✗	?	✗	✗	✗	?	?	✗	None	Chew et al. (2015) [64]
		66 repeats			✓	?	✓	✓	✓	?	?	✓	Severe	
Human C9orf72 BAC	BAC containing full *C9orf72* gene region (~36kb) with ~110kb upstream and ~20kb downstream	Unknown copy number; ~100-600 repeats (F112)	C57BL/6J	Male mice examined. No behavioural or motor abnormalities.	✓	✓	✓	?	?	?	?	✗	None	O'Rourke et al. (2015) [65]
		Unknown copy number; ~100-1000 repeats (F113)			✓	✓	✓	?	?	?	?	✗	?	
		Unknown copy number; 15 repeats			✗	✗	✗	?	?	?	?	✗	?	
Human C9orf72 BAC	BAC containing 140.5kb upstream and exons 1-5 of the C9orf72 gene	2 copies; 300 repeats/500 repeats	SJL/B6	Male mice examined. No behavioural or motor abnormalities. No evidence of neuronal dysfunction.	✓	✓	✓	?	?	?	?	✗	None	Peters et al. (2015) [66]
Human C9orf72 BAC	BAC containing 140kb upstream and exons 1-5 of the C9orf72 gene	1 copy; 450 repeats (high expression)	C57BL6/CH3 injected, backcrossed into C57BL/6	No motor abnormalities in any tested line. Male but not female medium and high 450 repeat expressors display cognitive and behavioural dysfunction.	✓	✓	✓	✓	✓	✗	✗	✗♦	Mild	Jiang et al. (2016) [36]
		1 copy; 450 repeats (medium expression)			✓	✓	✓	?	?	✗	✗	?	Mild	
		1 copy; 450 repeats (low expression)			✓	✓	✓	?	?	✗	✗	?	?	
		1 copy; 110 repeats (medium expression)			✗	✗	✗	?	?	✗	✗	?	None	
Human C9orf72 BAC	BAC containing full 29.6kb *C9orf72* gene with ~52kb upsteam and ~19.4kb downstream sequence (~98kb total)	1 copy: 500 repeats	FVB/NJ	Male and female mice examined. Either acute phenotype, slower progressive phenotype (symptomatic mice), or asymptomatic. Acute phenotype characterised by reduced survival. Symptomatic mice display behavioural and motor abnormalities.	✓	✓	✓	✓	?	?	?	✓	Severe in acute end stage animals. Moderate in slow progressive animals. None in asymtomatic animals.	Liu et al. (2016) [67]
		2 copies: 500 repeats/32 repeats			✓	✓	✓	✓	?	?	?	✓		
		4 copies: 36/29 repeats			✗	✗	✓	✓	?	?	?	✓		
		1 copy: 37 repeats		Male and female mice examined, no phenotypes observed	✗	✗	✗	✗	?	?	?	✗	None	

Table 2: Comparison of mouse models of *C9orf72* repeat expansion.

✓=Detected, x=Not detected, ?=Not examined

x♦Increased phospho-TDP43 observed by immunoblot vs non-transgenic, but no mislocalisation observed.

Study	Model system	GR	PR	GA	AP	GP
Kwon et al. (2014) [80]	U2OS cells / human astrocytes	20*	20*			
May et al. (2014) [72]	HEK293 cells	149	175	175	175	80
Mizielinska et al. (2014) [61]	*Drosophila*	36; 100	36; 100	36; 100	36; 100	
Wen et al. (2014) [59]	*Drosophila*		50	50	50	
Wen et al. (2014) [59]	Mouse primary neurons	25-400	25-200	25-400	25-200	25; 50
Wen et al. (2014) [59]	Human iPSC derived neurons		50	50		
Freibaum et al. (2015) [62]	*Drosophila*	50		50		47
Jovičić et al. (2015) [81]	*Saccharomyces cerevisiae*	50;100	50	50	50	
Tao et al. (2015) [75]	HEK293 / NSC34 cells	30	30	30	30	30
Yamakawa et al. (2015) [84]	Neuro-2a cells	100	100	100	100	100
Yang et al. (2015) [85]	*Drosophila*	80	80	80		
Boeynaems et al. (2016) [82]	*Drosophila*	50	25; 50	50	50	
Kanekura et al. (2016) [76]	NSC34 cells	20*	20*	20*		
Lopez-Gonzalez et al. (2016) [77]	Human iPSC derived motor neurons	80		80		
Lee et al. (2016) [79]	Neuro-2a cells	50	50	50	50	47
Lee et al. (2016) [79]	*Drosophila*	50	50	50	50	47

Legend:
- Toxic
- Mild or length dependent toxicity
- Not toxic
- Not tested

Table 3: Comparison of studies that compare the toxicity of individual DPRs in different model systems.

Numbers indicate the repeat length(s) tested in the study, note: in some studies the dipeptide proteins are epitope tagged.

*Peptides were exogenously applied to cells rather than overexpressed. Note poly-GA was found to be toxic in primary neurons [72–75] but these studies were excluded from the table as they do not compare to other DPRs.

Acknowledgements

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