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Modelling motor neuron disease in fruit flies: Lessons from spinal muscular atrophy

Beppe Aquilina[a,b], Ruben J. Cauchi[a,b,*]

[a] Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta
[b] Centre for Molecular Medicine and Biobanking, Biomedical Sciences Building, University of Malta, Msida, Malta

HIGHLIGHTS

- Genetic factors play a major role in MND pathogenesis and progression.
- SMA, caused by mutations in the *SMN1* gene, leads to muscle weakness and paralysis.
- Fruit fly models of SMA have a phenotypic overlap with patients and mammalian models.
- Discovery of genetic and chemical modifiers can lead to complementary SMA therapies.
- SMA fly models pave the way for MND modelling in *Drosophila*.

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ABSTRACT

Motor neuron disease (MND) is characterised by muscle weakness and paralysis downstream of motor neuron degeneration. Genetic factors play a major role in disease pathogenesis and progression. This is best underscored by spinal muscular atrophy (SMA), the most common MND affecting children. Although SMA is caused by homozygous mutations in the *survival motor neuron 1* (*SMN1*) gene, partial compensation by the paralogous *SMN2* gene and/or genetic modifiers influence age of onset and disease severity. SMA is also the first MND that is treatable thanks to the recent development of a molecular-based therapy. This key milestone was possible following an intense research campaign in which animal models had a starring role. In this review, we specifically focus on the fruit fly *Drosophila melanogaster* and highlight its sterling contributions aimed at furthering our understanding of SMA pathogenesis. Methods of gene disruption utilised to generate SMA fly models are discussed and ways through which neuromuscular defects have been characterised are elaborated on. A phenotypic overlap with patients and mammalian models, allowed the use of SMA fly models to identify genetic modifiers, hence spurring investigators to discover pathways that are perturbed in disease. Targeting these can potentially lead to complimentary therapies for SMA. The same output is expected from the use of SMA fly models to identify therapeutic compounds that have an ameliorative effect. We believe that lessons gained from SMA will allow researchers to eagerly exploit *Drosophila* to confirm novel genes linked to MND, reveal disease mechanisms and ultimately identify therapeutics.

1. Introduction

Motor neuron disease (MND) is an umbrella term for a collection of clinically and aetiologically heterogeneous group of neurological conditions that are characterised by muscle weakness thought to arise from the selective degeneration of lower and/or upper motor neurons. MND causes rapidly progressive motor dysfunction ultimately leading to premature death following respiratory failure in the majority of cases. Genetic factors undoubtedly are major players in disease pathogenesis and progression (Dion et al.,

Abbreviations: ALS, amyotrophic lateral sclerosis; CB, Cajal body; CNS, central nervous system; LOF, loss of-function; NMJ, neuromuscular junction; PRMT5, protein arginine methyltransferase 5; RNAi, RNA interference; SMA, spinal muscular atrophy; SMN, survival motor neuron; snRNP, small nuclear ribonucleoprotein; Tgs1, trimethylguanosine synthase 1; UAS, upstream activation sequence.

* Corresponding author at: Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta.
E-mail address: ruben.cauchi@um.edu.mt (R.J. Cauchi).

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2009; Taylor et al., 2016). This is highlighted by spinal muscular atrophy (SMA), the most common MND striking infants. SMA is typically an autosomal recessive disorder caused by inactivating mutations of the *survival motor neuron 1* (*SMN1*) gene that are partially compensated by the paralogous *SMN2* gene. SMA is therefore caused by a reduction rather than total loss of the *SMN1/2*-encoded SMN protein, with *SMN2* copy number influencing age of onset, disease severity and progression rate throughout the lifespan. To this end, SMA is clinically classified as type I (severe), II (intermediate), III (mild) or IV (adult-onset) (Burghes and Beattie, 2009; Kolb and Kissel, 2011). The successful development of a molecular-based therapy for SMA, essentially repairing the *SMN2* gene (Talbot and Tizzano, 2017), provides a roadmap for the engineering of therapeutics tailored for other hereditary motor neuron degenerative conditions including the most prevalent MND of adulthood, amyotrophic lateral sclerosis (ALS). Intense research on the molecular genetics underpinning such disorders followed by functional characterisation of the disease-associated genes in animal models are key steps that provide a very good starting point for this challenging endeavour.

The fruit fly *Drosophila melanogaster* is often the perfect choice of model organism to model neurodegenerative conditions for several reasons (Cauchi and van den Heuvel, 2006; Lessing and Bonini, 2009; McGurk et al., 2015). First, *Drosophila* has a rapid life cycle with adults emerging after ~10 days. In addition, its short lifespan (2–3 months) is considered an asset in the context of neurodegenerative disease research. Cost-intensive handling and maintenance, characteristics of vertebrate model organisms do not factor in *Drosophila* husbandry and ethical restrictions do not apply. Second, since flies were applied to biological research more than a century ago by Thomas Hunt Morgan to discover the role of chromosomes in heredity, a rich genetic toolbox has been developed, allowing researchers to easily manipulate and control *Drosophila* genetics. A colossal number of mutant and transgenic fly lines have been generated over the years. In view that *Drosophila* stocks cannot be frozen, they are maintained alive in laboratories all over the world and in 'public libraries' including the Bloomington *Drosophila* Stock Centre (BDSC) at Indiana University (Cook et al., 2010), the Kyoto Stock Centre at the Kyoto Institute of Technology and the Vienna *Drosophila* Resource Center (Dietzl et al., 2007). Stocks are commonly shared between laboratories or purchased at a low-cost from libraries. Researchers also have access to volumes of curated data on every single *Drosophila* gene on the FlyBase database (flybase.org). Third, comparison of the fly and human genomes exposed a significant overlap or conservation in genes and pathways (Rubin et al., 2000), with nearly 75% of known human disease-linked genes having homologues in flies (Reiter et al., 2001). In this setting, expression of the disease form of the human gene or mutations in its *Drosophila* homologue allow researchers to generate fly models of human disease with the aim of discovering mechanisms underpinning the disease process. Furthermore, the compact nature of the *Drosophila* genome and the relatively lower level of genetic redundancy facilitate exploration of gene function. Fourth, flies are able to respond rapidly to stimuli in addition to performing complex motor behaviours including climbing and flight because they are endowed with a sophisticated neuromuscular system, that though simpler relative to humans, has the basic sensory-motor circuitry, glia and multinucleate muscle fibres (Lloyd and Taylor, 2010). This feature is key for neuromuscular disease modelling and considering the remarkable conservation at a genetic, molecular, and physiological level the fly is increasingly considered as instrumental for gaining insights into the function of novel genes linked to MND in the era of whole-genome sequencing. In this review, we will focus on how *Drosophila* was harnessed to better understand SMA considering that the strategies undertaken (summarised in Fig. 1) can serve as a paradigm for the unravelling of molecular mechanisms underpinning emerging hereditary neuromuscular conditions.

2. Loss-of-function approach

Loss-of-function (LOF) gene manipulation is usually the first step undertaken following identification of the *Drosophila* orthologue of the gene involved in human disease. In *Drosophila*, genes can be disrupted via various methods including unbiased ethyl methanesulfonate (EMS) mutagenesis, transposable element insertion, insertional/replacement homologous recombination and gene editing via programmable nucleases (reviewed in Ref. Lin et al., 2014). Phenotypes associated with either complete loss of the gene of interest (amorph) or a reduction in its function (hypomorph) provide key information on the function of the encoded protein. Hence, the first *Drosophila* study on SMN investigated LOF missense mutations in the coding region of the fly *SMN* orthologue (Chan et al., 2003). Similar mutations present in the C-terminal YG-box of SMN, were reported in several SMA patients and result in a protein that is defective in self-binding (reviewed in Ref. Burghes and Beattie, 2009). In flies, such alleles were found to induce motor dysfunction at an early stage of development in homozygotes (Chan et al., 2003). Mutant SMN^{73Ao} larvae had reduced mobility, and, on dissection, they showed substantial defects at the neuromuscular junction (NMJ) including disorganised boutons and decreased expression of neurotransmitter receptors (Chan et al., 2003; Lee et al., 2008). *Drosophila* allows investigators to easily probe the tissue-specific requirements of a gene. The strategy that is most often employed involves the GAL4/*Upstream Activation Sequence* (*UAS*) system to express a transgene in a mutant background using tissue-specific drivers (Brand and Perrimon, 1993; Duffy, 2002). Making use of this approach, *SMN* gene add-back in both neuronal and muscle tissue, but not in either, was required to revert the larval lethality of *SMN* mutant flies (Chan et al., 2003). Investigation of homozygous *SMN* null mutants (SMN^{X7}), which are flies that have a small deficiency that removes the entire *SMN* coding region, revealed that, in addition to defective locomotion, flies also have reduced muscle size, aberrant rhythmic motor output and defective NMJ neurotransmission (Imlach et al., 2012). Interestingly, restoration of SMN specifically in both proprioceptive neurons and cholinergic interneurons in the motor circuit was sufficient to correct these defects. Although the origin of the motor system deficits in SMA remains debatable, *Drosophila*-based studies point to the motor unit encompassing the sensory-motor circuit and muscle as being the most vulnerable to SMN deficiency. This is in line with studies in vertebrate models (Cifuentes-Diaz et al., 2001; Gavrilina et al., 2008; Gogliotti et al., 2012; Hua et al., 2015; Hunter et al., 2014, 2016; Jablonka et al., 2006; Martinez et al., 2012; Passini et al., 2010; Rindt et al., 2015). Importantly, SMN depletion in flies resulted in phenotypes that overlap with those observed in SMA patients (Burghes and Beattie, 2009; Lefebvre et al., 1997). This confirmed that SMN is the causative gene for SMA in addition to supporting the continued use of *Drosophila* as a model organism for this monogenic MND.

The delayed lethality of SMN mutants is due to maternally-derived wild-type SMN, which is present at low levels and, hence, mimics the situation in SMA patients. Indeed, complete loss of SMN is incompatible with life in a multitude of model organisms including yeast, worm, zebrafish and mouse (Edens et al., 2015). Nonetheless, *Drosophila* allowed researchers to capture the earliest cellular phenotypes resulting from total ablation of SMN. This analysis was possible following examination of germ-line clones homozygous for the SMN^{73Ao} allele making use of the Flp/*FRT* system. This ingenious genetic tool makes use of the yeast-derived Flp recombinase to mediate efficient site-specific recombination

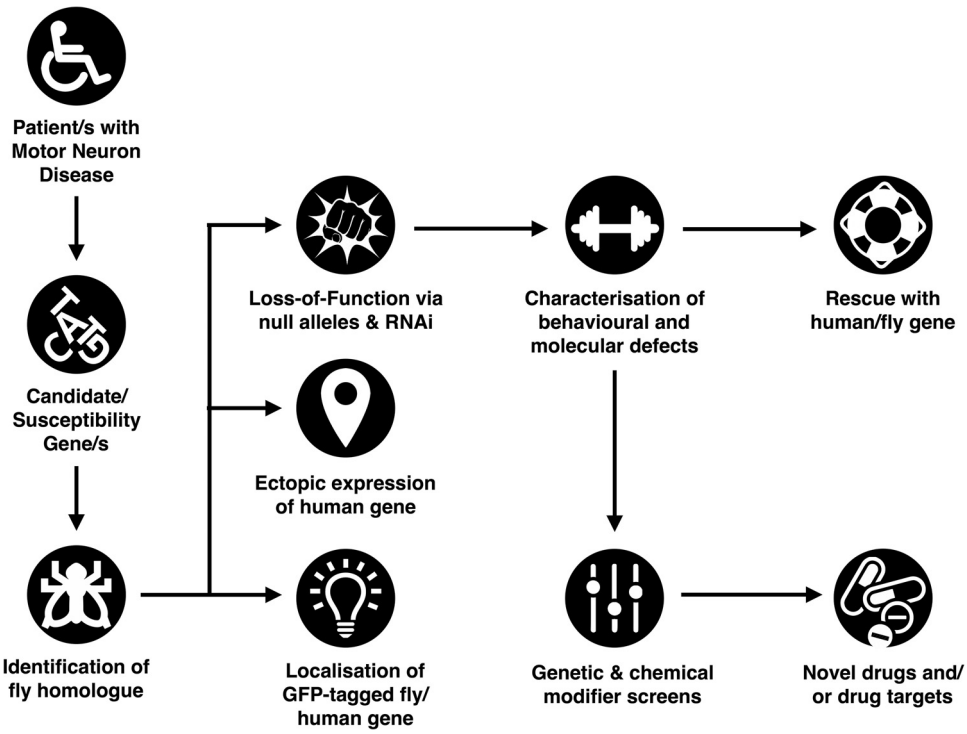

Fig. 1. Research strategy for generating and characterising a *Drosophila* model for a human motor neuron disease. Discovery of disease-linked genes identified through genetic studies including whole-genome or exome sequencing allows researchers to manipulate the fly orthologue. This is mainly done through loss-of-function, specifically the generation of amorphic or hypomorphic alleles with the latter including RNAi transgenes to knockdown gene expression. Gain-of-function studies are also possible through the use of the bipartite GAL4/*UAS* system, which is commonly used to express transgenes including those with a dominant-negative effect or the human version of the gene under investigation. Introducing human mutations within the fly orthologue under endogenous regulatory control is also a common approach. Resulting phenotypes including behavioural and molecular deficits are characterised and compared to those associated with the human condition and other models. Significant overlap confirms the gene as the causative factor for the disease studied in addition to establishing a fly model of the disease. Rescue of phenotypes with the human gene underscores functional conservation. The identification of genetic modifiers through candidate or genome-wide screens helps researchers not only gain insights into disease pathways and mechanisms but also identify novel druggable gene targets. Fly models can also be used to single out pharmacological agents that suppress the disease phenotype.

between transgenic *FRT* (Flp recombinase target) sites present at identical positions on homologous chromosomes, hence generating a mutagenized chromosome arm homozygous in clones of cells. The application of the dominant female sterile (DFS) technique to the Flp/*FRT* system, which makes use of the dominant ovo^D mutation to kill the non-recombinant germ cells, further allowed the researchers to generate female flies that only lay eggs derived from homozygous SMN^{73Ao} mutant germ-line clones. Oocytes from these females, which are devoid of wild-type SMN activity rarely reached maturity and when they did and were fertilised, the embryonic lethality was not rescued by the paternal wild-type chromosome (Chan et al., 2003). Defective nuclear organisation was found out to be the most prominent early defect in homozygous SMN^{73Ao} mutant eggs, hence pointing to the involvement of SMN in maintaining the structure of chromosomes and nuclear organelles including nucleoli, Cajal bodies (CBs) and histone locus bodies (Lee et al., 2009). Similar phenotypes were observed in egg chambers mutated for the SMN-associated protein Gemin3 (Cauchi, 2012). Interestingly, DNA damage is also an outcome of SMN deficiency in a recently described mouse model of SMA (Jangi et al., 2017).

Biochemical studies have shown that SMN is crucial for the assembly of small nuclear ribonucleoproteins (snRNPs), which are the operating components of the spliceosome. This process essentially entails the coupling of a core of seven Sm proteins to small nuclear RNAs (snRNAs) (Fischer et al., 2011; Kolb et al., 2007). The major spliceosome, a conglomerate of U1, U2, U4/U6 and U5 snRNPs, catalyses the removal of the majority of pre-mRNA introns. The less abundant minor spliceosome, which processes a rare non-canonical group of introns is however comprised of U11, U12, U4atac/U6atac and U5 snRNPs (reviewed in Ref. Patel and Steitz, 2003). *Drosophila* is perfectly suited to translate *in vitro* findings, and in case of SMA, it exposed the mechanism linking deficiency of a ubiquitous spliceosome assembly factor to selective motor dysfunction. To this end, SMN^{73Ao} mutant larvae were found to have decreased levels of snRNAs (Lotti et al., 2012), a result that confirmed earlier findings in SMN^{73Ao} mutant clones within the developing fly central nervous system (CNS) (Grice and Liu, 2011). Downstream consequences included perturbed splicing and decreased expression of a subset of minor-class intron-containing genes. The latter is thought to occur because under normal conditions minor-class intron splicing occurs at a slower rate that is exacerbated when availability of minor spliceosome snRNPs is reduced in an SMN deficient background. *Stasimon*, which encodes for an evolutionarily conserved transmembrane protein required for motor circuit function, is one of the minor-class intron-containing genes which shows altered expression when SMN levels are reduced. Interestingly, loss of *stasimon* resulted in NMJ phenotypes that overlapped those ascribed to SMN deficiency, and restoration of Stasimon expression in the motor circuit of SMN^{X7} null mutant flies rescued muscle growth as well as neurotransmitter release at the NMJ. Similar results in zebrafish and mouse models of SMA indicate that *stasimon* is a universal SMN target gene whose expression can be therapeutically manipulated to ameliorate motoric abilities in SMA patients (Lotti et al., 2012). Notwithstanding studies that argue against a minor-intron-dependent aetiology for SMA (Garcia et al., 2013), such findings corroborate increasing evidence linking SMN-dependent snRNP defects to motor circuit dysfunction *in vivo* (reviewed in Ref. Lanfranco et al., 2017b).

Gene knockdown, typically done through spatiotemporally controlled expression of RNAi against the gene of interest, can also be informative. Although one needs to develop strategies to ensure specificity and efficacy, RNAi offers the possibility of developing hypomorphic alleles of the gene under investigation. Making use of the versatile GAL4/*UAS* system to express SMN RNAi constructs in either muscle or neurons, investigators have determined that, in line with earlier studies (above), SMN function is required in both tissues for organismal survival and locomotor ability (Chang et al., 2008; Timmerman and Sanyal, 2012). Behavioural deficits were replicated if SMN knockdown was selectively restricted to glutamatergic neurons including motor neurons (Timmerman and Sanyal, 2012). Importantly, either muscle- or neuron-specific SMN knockdown resulted in flies with prominent NMJ defects at the larval stage, thereby suggesting that NMJ morphology is dependent on both pre- and post-synaptic SMN activity (Chang et al., 2008). Interestingly, imprecise excision of a transposable element in the promoter region of the *SMN* gene allowed researchers to isolate a hypomorphic SMN mutant (SMN^{E33}) that shows marked defects in flying and jumping, thereby developing an adult model of SMA. Homozygous SMN^{E33} mutants have reduced SMN levels in the adult thorax that leads to muscle atrophy as well as pronounced motor neuron routing and arborisation defects, which are phenotypes seen in vertebrate models of SMA or SMA patients themselves (Rajendra et al., 2007). In addition to generating novel fly models of SMA, we predict that the availability of easy-to-use gene editing techniques, mainly the CRISPR/Cas9 system, will revolutionise the art of neuromuscular disease modelling in the fly.

3. Interaction profiling

Discovery of protein-protein interaction networks is key for unravelling the function of a disease-associated protein and for exploring disease mechanisms. Considering SMN, molecular and structural studies have shown that it operates as part of a large multimeric complex whose components also include a set of diverse proteins, namely Gemins 2–8 and Unrip (Cauchi, 2010). In a recent report, we found that the *Drosophila* SMN complex is fully conserved, hence, it has the same number of components and is organised in a similar fashion to that present in vertebrates including humans (Lanfranco et al., 2017a). Importantly, components of the *Drosophila* SMN complex were shown to interact physically and/or genetically (Borg and Cauchi, 2014; Borg et al., 2015; Cauchi et al., 2008; Guruharsha et al., 2011; Kroiss et al., 2008; Lanfranco et al., 2017a; Shpargel et al., 2009). The latter demonstrates that *Drosophila* is a favourite model organism for the conduction of candidate-based genetic-modifier studies. Interestingly, our laboratory has also found that with the exception of Gemins 6 and 7, disruption of any Gemin component or Unrip leads to flies with neuromuscular deficits, thereby phenocopying SMN disruption (Borg and Cauchi, 2013; Borg et al., 2016; Cauchi et al., 2008; Lanfranco et al., 2017a). This means that the majority of SMN complex components are critical for normal motor behaviour *in vivo* since absence of any member is sufficient to arrest SMN complex function.

Probing the mechanics of SMN complex formation in *Drosophila* also proved to be an informative endeavour. Zygotic expression of *SMN* transgenes with YG-box missense mutations has a severe effect on homozygous SMN^{X7} null larvae that is suppressed on overexpression of a wild-type *SMN* transgene (Praveen et al., 2014). It is highly-likely that oligomerisation-defective SMN has a dominant-negative effect on wild-type (maternally-contributed) SMN and/or its Gemin associates within the SMN complex. Our laboratory has also uncovered dominant-negative interactions when a Gemin3 transgene lacking the helicase domain ($Gem3^{\Delta N}$) is expressed in flies (Cauchi et al., 2008). We and others have also showed that an imbalance in the protein levels of its constituents can have a destabilising effect on the SMN complex. Hence, in addition to SMN, reduced levels of Gemins 2, 3, 4, 5, 8 or Unrip is deleterious in *Drosophila* (Borg and Cauchi, 2013; Borg et al., 2016; Chang et al., 2008; Gates et al., 2004; Lanfranco et al., 2017a; Shpargel et al., 2009) and this is in agreement with results from *in vitro* studies done earlier (Ogawa et al., 2007; Shpargel and Matera, 2005). On investigating gain-of-function effects, Gemin2 was found to have a toxic effect when overexpressed in wild-type flies, and this is a phenotype that is also seen in the fission yeast *Schizosaccharomyces pombe*. Upregulation of SMN or Gemin5 also impacts viability in flies expressing a Gemin3 hypomorphic mutant (Borg et al., 2015). Moreover, although it has no effect on fly survival, overexpression of SMN in wild-type flies, alters the timing of CNS and testis growth, and disrupts the onset of pupariation (Grice and Liu, 2011). These findings corroborate studies that highlight the interdependence of constituent levels within the SMN complex including those that show reduced protein levels of select Gemins in SMA cell cultures (Carissimi et al., 2006; Feng et al., 2005; Gabanella et al., 2007; Hao le et al., 2007; Helmken et al., 2003; Jablonka et al., 2002; Shpargel and Matera, 2005) or SMA mice (Gabanella et al., 2007; Zhang et al., 2008).

Assembly of snRNPs is not a task credited solely to the SMN complex. Indeed, the number of trans-acting assembly factors involved in this process add up to at least twelve, a count far greater than the parts to be assembled (Fischer et al., 2011). Hence, snRNP assembly entails cooperation between the SMN complex and the protein arginine methyltransferase 5 (PRMT5) complex, whose constituents include WD45, PRMT5 and pICln. PRMT5 induces post-translational modifications in select Sm proteins and ordered-organisation of Sm protein subsets is possible due to pICln. Upon hand-over of pICln-bound Sm proteins to Gemin2, and channeling of snRNAs via Gemin5, the SMN complex ensures stringent snRNP synthesis. ATP requirement of the SMN complex has been attributed to DEAD-box RNA helicase Gemin3, which might also be involved in RNP remodelling during snRNP biogenesis (Curmi and Cauchi, 2018). Import of snRNPs to the nucleus requires cap hypermethylation by the trimethylguanosine synthase I (Tgs1) (reviewed in Ref. Lanfranco et al., 2017b). In this context, it is interesting that SMN interacts genetically with *Dart5*, the *Drosophila* PRMT5 orthologue (Gonsalvez et al., 2008), whereas Gemin3 associates both genetically and physically with pICln and Tgs1 (Borg et al., 2016). Additional results from our laboratory show that disruption of either pICln or Tgs1, induces phenotypes that closely resemble those resulting from SMN complex perturbation (Borg et al., 2016). In this regard, findings in *Drosophila* seems to favour the possibility that a role for the SMN complex in snRNP biogenesis is crucial for the health of the neuromuscular system, and disturbance of this pathway is most likely to blame for the motor dysfunction seen in SMA patients.

Drosophila is also conducive to large-scale genetic-modifier screens which allow investigators to identify novel pathways involved in the disease process. To this end, to identify enhancers of SMN-associated lethality the Artavanis-Tsakonas lab at Harvard Medical School tested a collection of transposon-induced mutations that produce synthetic lethality or semi-lethality in an SMN^{73Ao} heterozygous background. In a second phase of the screen, they identified insertions that suppressed the larval lethality associated with homozygous SMN^{73Ao} mutant. It is noteworthy that in addition to loss-of-function, transposon insertions can also induce gain-of-function (hypermorph or neomorph) if they harbour *UAS* sequences. The latter can be induced to overexpress the downstream gene in the presence of a GAL4 transcription factor. By screening approximately 50% of the *Drosophila* genome, the researchers succeeded in identifying 27 SMN modifiers (Chang et al., 2008). Although the identified modifier genes have no obvious

role in snRNP assembly, their expression might be altered downstream to SMN attenuation. Some including several components of the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) signalling pathway were shown to alter the NMJ phenotype associated with reduced SMN levels in addition to survival (Chang et al., 2008; Sen et al., 2011). These studies suggest that targeting these two pathways in SMA patients by therapeutic agents may help to alleviate symptoms of the disease. In a later study, the same lab performed a complementary screen this time using a hypomorphic SMN RNAi allele to increase sensitivity. More than 300 candidate genes were identified. Integrating the genetic screen results with large-scale protein interaction studies and bioinformatic analysis, the authors succeeded in defining a unique SMN interactome that provides the basis for a better understanding of SMA. Additionally, they establish an integrated approach that can be applied to other neurodegenerative conditions (Sen et al., 2013).

Identification of conserved pathways relevant to SMA pathology can be accelerated through studies involving more than one model organism. To this end, combining *Drosophila* with *C. elegans* resulted in the identification of several cross-species modifier genes of SMN loss of function (Di Giorgio et al., 2017; Dimitriadi et al., 2010). These include the actin-binding and -bundling protein Plastin3, which was found to modulate SMA severity in earlier genetic studies on humans (Oprea et al., 2008), an outcome that increases confidence in the clinical relevance of genetic modifiers identified in invertebrate models of SMA. Plastin3 knockout in yeast impairs endocytosis (Kubler and Riezman, 1993), and disturbed endocytosis was later found to be a major cellular mechanism underlying impaired NMJ function and maintenance in SMA (Dimitriadi et al., 2016; Hosseinibarkooie et al., 2016). The identification of Neurocalcin Delta, a second SMA protective modifier in humans and, its ability to restores endocytosis in SMA models, underscores the possibility that the endocytic pathway can be targeted for therapy in patients (Riessland et al., 2017). Although they were not applied to SMA models, downregulation screens are also possible. One common strategy involves the use of the GAL4/*UAS* system to induce RNAi-mediated knockdown of every single gene within the genome to identify suppressor or enhancers of a disease-linked mutant phenotype. For instance, overexpression of the ALS-associated VAPB in sensory neurons leads to loss of thoracic bristles in *Drosophila*. An RNAi-based screen was successful in identifying several modifiers of this phenotype allowing researchers to build a genetic network for VAPB (Deivasigamani et al., 2014).

4. Ectopic gene expression

Through the use of the powerful GAL4/*UAS* system, *Drosophila* permits investigators to test if expression of human cDNA can correct the phenotypes associated with loss of a homologous fly gene. This was, for instance, the outcome achieved on expression of the ALS-associated *TDP-43* gene in mutants lacking the fly equivalent gene (Feiguin et al., 2009), hence indicating functional conservation. The same experiment was not successful for SMN (Borg and Cauchi, 2013), possibly because human SMN has a dominant-negative (antimorph) effect on maternally-derived wild-type *Drosophila* SMN that is still present in SMN mutant larvae. Indeed, ectopic expression of human SMN in wild-type flies induces pupal lethality due to the formation of non-functional fly:human SMN heteroligomers (Miguel-Aliaga et al., 2000). Nevertheless, researchers were not deterred from analysing the functional consequences of SMA patient variants. They did so by changing conserved amino acid sequences in the fly SMN homologue to mimic SMA disease variants. Using this strategy, expression of either wild-type *SMN* or a type II/III SMA variant (SMN^{T205I}, which mimics the human SMN^{T274I} variant) was found to rescue locomotor defects and larval lethality in SMN null mutants. However, snRNA levels were not restored to normal levels, a result indicating that loss of snRNP-independent roles for SMN might also contribute to the disease (Praveen et al., 2012). Considering the non-canonical functions ascribed to SMN, a role in axonal mRNP assembly and trafficking remains in pole position (Donlin-Asp et al., 2016; Fallini et al., 2012). Axonal transport defects are a common phenotype in *Drosophila* models of ALS (Baldwin et al., 2016) and this might also be the case for SMA. To this end, SMN^{73Ao} homozygous mutant neuroblasts failed to correctly localise Miranda at the basal membrane (Grice and Liu, 2011). Miranda is known to form a complex with RNA-binding protein Staufen, which binds and transports several mRNPs in neuroblasts and axons (Roegiers and Jan, 2000; Tosar et al., 2012). Nonetheless, further *Drosophila*-based studies examining the axonal role of SMN are warranted.

In addition to rescue analyses, the GAL4/*UAS* binary expression system is also typically applied to express epitope-tagged transgenes, thereby allowing researchers to probe the sub-cellular localisation of the gene product under investigation. To this end, the use of an SMN transgene tagged with yellow fluorescent protein (YFP) was a key tool for characterising discrete snRNP-rich cytoplasmic bodies termed U bodies (Liu and Gall, 2007). These organelles were later found to contain not only SMN but also Gemins 2, 3 and 5, hence, the full SMN complex known at that time (Cauchi et al., 2010). To conduct cell biology studies *in vivo*, researchers including ourselves often take advantage of the large size offered by the cells in the easily-accessible egg chambers making up the *Drosophila* ovary. Nonetheless, U bodies are present in multiple *Drosophila* tissues and, importantly, such structures are universal in view of their presence in most eukaryotic cells including those derived from humans (Cauchi, 2010; Liu and Gall, 2007). Whether U bodies are sites for snRNP assembly or storage depots of snRNPs remains an unanswered question. Nonetheless, even in times of stress, U bodies are invariably associated with P bodies, which are involved in RNA surveillance and decay (Buckingham and Liu, 2011; Liu and Gall, 2007). Furthermore, disruption of one body affects the organisation of the other, suggesting that the two cytoplasmic organelles might cooperate in regulating aspects of RNA metabolism (Lee et al., 2009; Liu and Gall, 2007). SMN localisation was also crucial for the discovery of the elusive *Drosophila* CB (Liu et al., 2006, 2009), and overexpression of SMN complex component Gemin3, in *Drosophila*, exposed nuclear gems, which are often found associated with CBs (Cauchi, 2011; Liu and Dreyfuss, 1996). Whereas the CB is known to host several important steps in the maturation of the RNA-processing machinery (reviewed in Ref. Stanek, 2016), studies in *Drosophila* support the hypothesis that gems are storage sites for excess nuclear SMN complexes that can be readily recruited for nuclear ribonucleoprotein assembly reactions occurring in CBs (Cauchi, 2011). Depletion of gems is a signature feature of both SMA and ALS (Cauchi, 2014).

One should be aware that the detected signals from fluorescently-tagged transgenes might be artefacts of overexpression. To eliminate this pitfall, antibodies should be generated against the gene product of interest and used to stain tissues derived from wild-type flies. In this regard, antibodies against SMN and the Gemin components of the SMN complex stained cytoplasmic foci in the same pattern as the respective tagged-transgene (Cauchi et al., 2010; Lee et al., 2009; Liu and Gall, 2007). Prompted by the link to MND, the localisation of the respective gene product within the neuromuscular system should be investigated. For instance, in muscle, SMN was found to localise within the sarcomere (Rajendra et al., 2007) and in post-synaptic regions of the NMJ (Chang et al., 2008), though such staining patterns still need to be independently confirmed. In the brain, SMN is enriched in postembryonic neuroblasts and forms a concentration gradient in the differentiating progeny including motor neurons (Grice and Liu, 2011). Antibody production remains a time-consuming and

expensive service without a guarantee of success, so the use of fluorescently-tagged transgenes still remains the swiftest and easiest path to discovering the whereabouts of the gene-product of interest.

5. Drug discovery

Although *Drosophila* has traditionally been a 'powerhouse' for genetic studies, this premier model organism is finding itself to be central for searching therapeutic compounds that have an ameliorative effect on various diseases including the neurodegenerative spectrum. Compounds that are originally known for their activity in human cells were shown to have the same molecular mechanism of action in *Drosophila* (Fernandez-Hernandez et al., 2016), a finding that bodes well for the application of *Drosophila* in drug discovery. Considering that *Drosophila* is still a new tool in this field, studies that make use of this approach for SMA are in their infancy. The McCabe lab for instance investigated whether pharmacological antagonists of K^+ channels could correct SMN mutant phenotypes in *Drosophila* considering that genetic methods that inhibit K^+ channel activity were successful in this regard. 4-Aminopyridine (4-AP), an FDA/EMA-approved small-molecule inhibitor of voltage-activated K^+ channels, was found to increase muscle surface area, and locomotor ability. Defects in rhythmic motor output and NMJ neurotransmission were also substantially improved (Imlach et al., 2012). These promising results prompted the investigators to conduct a Phase II/III study to test whether 4-AP, which is clinically used to treat multiple sclerosis (Chwieduk and Keating, 2010), can improve walking ability and endurance in adult patients with SMA type III. In another study, Wishart et al. (2014) observed widespread perturbations in ubiquitin homeostasis in mouse models of SMA including reduced levels of the ubiquitin-like modifier activating enzyme 1 (UBA1) and β-catenin accumulation. Interestingly, mutations in the gene coding for the human UBA1

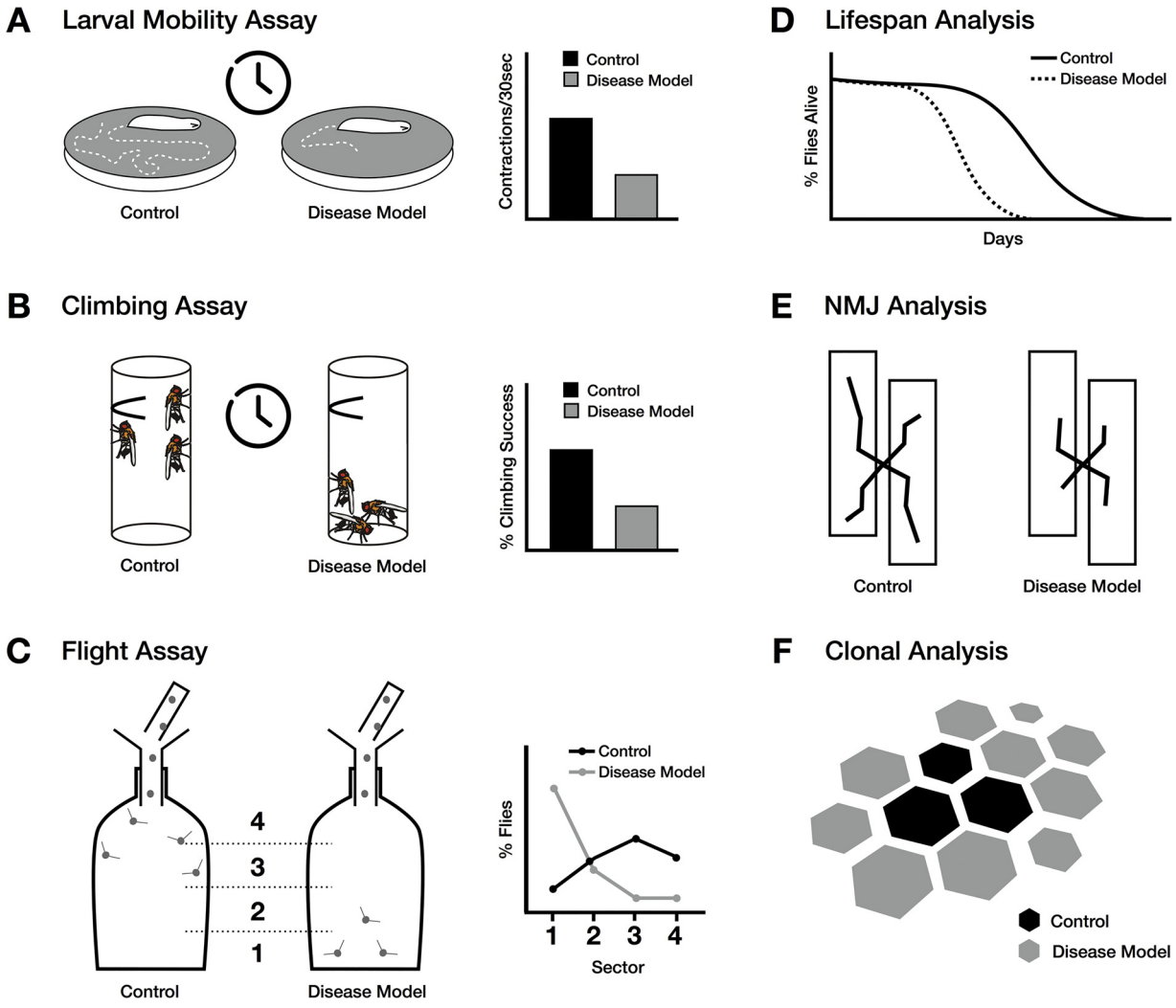

Fig. 2. Selection of robust assays that are used to assess neuromuscular dysfunction in *Drosophila*. (**A**) For assessment of motoric ability in larvae, the rate of forward body wall contractions is determined. This is typically done by counting the number of contractions exhibited by larvae in 30 s. (**B**) In adults, motor defects are typically examined by the negative geotaxis or climbing assay whereby flies are tapped to the bottom of an empty vial, and the number of flies that reach a pre-established threshold is recorded. Calculation of the percentage climbing success entails dividing the number of flies whose climbing was successful by the total number of flies and multiplying by 100. (**C**) In a flight assay, also used to determine motor deficits, flies are introduced into the top of an oil-coated cylinder. The number of flies stuck in each sector is then counted, divided by the total number of flies assessed and multiplied by 100 to generate the percentage number of flies per sector. Flight ability is determined by the height or sector in which flies are distributed, hence, non-fliers mostly accumulate in the lower sectors. (**D**) Lifespan analysis can be used to assess the survival of adult fly models, which is typically reduced in the presence of neuromuscular dysfunction or degeneration. Typically, surviving flies are counted every few days throughout their adult life. (**E**) Fly larvae can be easily dissected and stained with pre-synaptic and post-synaptic markers to visualise NMJ abnormalities including a reduction in bouton numbers. (**F**) To bypass embryonic phenotypes, investigators can generate mutant clones in brain, germline or imaginal discs. This approach allows cell biology studies in pockets of cells that are homozygous for the mutagenized chromosome, whereas the rest of the tissue is heterozygous.

can cause X-linked infantile SMA (Ramser et al., 2008). Importantly, pharmacological inhibition of β-catenin with quercetin, a plant-derived flavonoid, was found to ameliorate neuromuscular pathology in mouse, zebrafish and *Drosophila* models of SMA. Specifically, in *Drosophila*, quercetin treatment reversed NMJ morphological defects in a dose-dependent manner in SMN mutants (Wishart et al., 2014). In addition to highlighting β-catenin as a potential therapeutic target for SMA, this study underscores the power of an integrative strategy to disease modelling. Hence, combining the fly model system with other model systems will most probably become the mainstream approach to rapidly ascertain disease-associated pathways that are likely to be affected in human patients. Furthermore, taking advantage of the wealth of knowledge gained from the conduction of large-scale genetic screens, *Drosophila* models of disease are expected to be increasingly used for living organism-based high-throughput chemical screens.

6. Conclusion & prospects

By focusing on the sterling contributions of the fly model system to the SMA field, in this review we have given a flavour of the types of genetic, molecular and pharmacologic studies that are possible in *Drosophila*. SMA fly models mirror with remarkable similarities their mammalian counterparts and, importantly, the human condition. Here, we also touched upon several transferable robust assays that were used for SMA modelling (Fig. 2). The power of the fly is unique in that it allows investigators to identify modifiers with great ease and, in so doing, uncover in an unbiased manner the pathways that are perturbed in disease. The revolution in human genomic sequencing technologies will provide genetic findings that can be rapidly confirmed in *Drosophila*. In one recent example, *Drosophila* was instrumental to test the validity of a homozygous missense mutation, changing a conserved amino acid, in the *ATG5* gene identified through whole exome sequencing of two siblings with congenital ataxia, mental retardation and developmental delay. Indeed, flies in which the equivalent *ATG5* gene is substituted with the mutant human version were found to exhibit severe movement deficits, in contrast to flies expressing the wild-type human protein (Kim et al., 2016). Whilst these findings confirm that a mutation in *ATG5* can be responsible for ataxia, the fly model can now be used to further the understanding of this novel syndrome in addition to identifying modifying factors including pharmacological agents that have an ameliorative effect on the phenotype. We predict that *Drosophila* will be able to provide functional support for findings derived from the application of whole-genome sequencing to motor neuron disorders with an as yet unidentified cause. Furthermore, the ever-evolving tool kit available to manipulate the fly genome, including more sophisticated ways to generate humanized fly models through the application of CRISPR/Cas9 system (Bassett and Liu, 2014) and novel ways to manipulate genes (Senturk and Bellen, 2017) ensures that the fly is moving with the times. In this regard, *Drosophila* will continue to remain a relevant model system to reveal mechanisms underpinning disease thereby allowing a deeper understanding of human biology. The ultimate aim is the discovery of therapeutics that mitigate motor neuron disease, considered to be one of the most catastrophic disorders of the brain. Continued research using *Drosophila* will strengthen this resolve.

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