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Disruption of Survival Motor Neuron in Glia Impacts Survival but has no Effect on Neuromuscular Function in *Drosophila*

Marija Farrugia, [a,b] **Neville Vassallo** [a,b] **and Ruben J. Cauchi** [a,b*†]

[a] *Centre for Molecular Medicine and Biobanking, Biomedical Sciences Building, University of Malta, Msida, Malta*

[b] *Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta*

Abstract—Increasing evidence points to the involvement of cell types other than motor neurons in both amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA), the predominant motor neuron disease in adults and infants, respectively. The contribution of glia to ALS pathophysiology is well documented. Studies have since focused on evaluating the contribution of glia in SMA. Here, we made use of the *Drosophila* model to ask whether the survival motor neuron (Smn) protein, the causative factor for SMA, is required selectively in glia. We show that the specific loss of Smn function in glia during development reduced survival to adulthood but did not affect motoric performance or neuromuscular junction (NMJ) morphology in flies. In contrast, gain rather than loss of ALS-linked TDP-43, FUS or C9orf72 function in glia induced significant defects in motor behaviour in addition to reduced survival. Furthermore, glia-specific gain of TDP-43 function caused both NMJ defects and muscle atrophy. Smn together with Gemins 2–8 and Unrip, form the Smn complex which is indispensable for the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). We show that glial-selective perturbation of Smn complex components or disruption of key snRNP biogenesis factors pICln and Tgs1, induce deleterious effects on adult fly viability but, similar to Smn reduction, had no negative effect on neuromuscular function. Our findings suggest that the role of Smn in snRNP biogenesis as part of the Smn complex is required in glia for the survival of the organism, underscoring the importance of glial cells in SMA disease formation.

Key words: survival motor neuron, spinal muscular atrophy, *Drosophila*, Glia, SMN complex, snRNP biogenesis.

INTRODUCTION

RNA dysregulation is believed to be a key mechanism underpinning motor neuron disease (MND) (Ibrahim et al., 2012; Ito et al., 2017; Butti and Patten, 2018). In infants, spinal muscular atrophy (SMA) results from insufficient levels of the survival motor neuron (SMN) protein (Lefebvre et al., 1995). SMN together with Gemins 2–8 and Unrip forms the SMN complex which is indispensable for the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) (Cauchi, 2010; Lanfranco et al., 2017b). Essentially, this involves the uploading of a preorganised heptameric Sm protein core onto a conserved uridine-rich site within small nuclear RNAs in an ordered process restricted to the cell cytoplasm (Lanfranco et al., 2017b). Key factors are pICln which assembles Sm sub-cores in the early phase of the process (Chari et al., 2008; Grimm et al., 2013) and trimethylguanosine synthase 1 (Tgs1), which acts in the final stages to modify the cap of assembled snRNPs, a requirement for their import to the nucleus where they function (Mouaikel et al., 2002; Mouaikel et al., 2003). In adults, amyotrophic lateral sclerosis (ALS) is caused by highly penetrant variants residing in *Chromosome 9 open reading frame 72* (*C9orf72*), *transactive response DNA binding protein* (*TARDBP*) and *fused in sarcoma* (*FUS*) (Taylor et al., 2016). TAR DNA binding-protein 43 or TDP-43 (encoded by *TARDBP*), and FUS are RBPs that are involved in various aspects of RNA processing (Lagier-Tourenne et al., 2010; Ratti and Buratti, 2016). Expanded hexanucleotide repeats in *C9orf72* are known to be deleterious, via mechanisms that include intracellular RNA depositions that sequester essential RBPs and non-canonical translation into toxic dipeptide repeat (DPRs) (Balendra and Isaacs, 2018).

*Correspondence to: R. J. Cauchi, Centre for Molecular Medicine and Biobanking, Biomedical Sciences Building, University of Malta, Msida, Malta.
E-mail address: ruben.cauchi@um.edu.mt (R. J. Cauchi).
† ORCID: https://orcid.org/0000-0001-6150-1608.

Abbreviations: ALS, amyotrophic lateral sclerosis; C9orf72, Chromosome 9 open reading frame 72; Dcr-2, Dicer-2; DPR, dipeptide repeat; FUS, fused in sarcoma; Gem2, Gemin2; Gem3, Gemin3; Gem5, Gemin5; MND, motor neuron disease; NMJ, neuromuscular junction; PBS, phosphate buffered saline; SMA, spinal muscular atrophy; SMN, survival motor neuron; snRNP, small nuclear ribonucleoprotein; TARDBP, transactive response DNA binding protein; TDP-43, TAR DNA binding-protein 43; Tgs1, trimethylguanosine synthase 1.

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The last two decades have challenged the notion that MND is solely a disease of motor neurons. ALS is now thought to be a non-cell autonomous disease, with glia contributing significantly to the pathology of the disease (Philips and Rothstein, 2014). This is understandable given that glial cells provide structural and neurotrophic support for neurons in addition to being key players of neuroinflammation. Increasing evidence is also pointing to the involvement of cell types other than motor neurons in SMA pathology (Hamilton and Gillingwater, 2013). Studies have thus evaluated the contribution of glia in SMA. Astrogliosis has been observed in the spinal cord of both SMA patients and SMA mouse models (Rindt et al., 2015; Ohuchi et al., 2019). Microglia were shown to eliminate vulnerable SMA synapses and their depletion was shown to rescue the number and function of synapses, enhancing the motoric abilities of SMA mice (Vukojicic et al., 2019). Astrocyte cultures derived from the spinal cord of SMA mice are deficient in supporting both wild-type and SMN-deficient motor neurons (Martin et al., 2017), resulting in reduced synapse formation and synaptic transmission (Zhou et al., 2016). To this end, restoration of SMN specifically to astrocytes increased lifespan, improved gross motor function and rescued neuromuscular junction (NMJ) defects in severe SMA mice (Rindt et al., 2015). Although no benefit was seen in survival, selective correction of SMN protein levels in Schwann cells was found to nonetheless reverse myelination defects, improve neuromuscular function and ameliorate NMJ pathology in an SMA mouse model (Hunter et al., 2016). Importantly, replacement of SMN in both glia and motor neurons as opposed to solely in motor neurons was found to induce the greatest improvement in survival in SMA mice and, in some instances, a complete rescue was reported (McGovern et al., 2015).

Aiming at gaining further insights on the contribution of glia in SMA, here we made use of the *Drosophila* model system to evaluate whether Smn is required selectively in glia. We show that glial-selective loss of Smn function in an otherwise wild-type organism reduces survival to adulthood, an outcome that is similar to that achieved upon gain rather than loss of TDP-43, FUS or C9orf72 function. However, in contrast to gain of TDP-43, FUS or C9orf72 function, Smn reduction in glia had no deleterious consequences on neuromuscular function in flies. A similar pattern was observed upon perturbation of Smn complex components or disruption of pICln and Tgs1, two key snRNP biogenesis factors. Furthermore, whereas gain of TDP-43 function in glia induced defects at the neuromuscular junction (NMJ) of motor neurons and muscle atrophy, glia-selective Smn reduction had no major consequences on neuromuscular morphology. Our findings indicate that Smn is required in glia for survival rather than neuromuscular function in *Drosophila*.

EXPERIMENTAL PROCEDURES

Flies

Flies were cultured on food consisting of sugar, corn meal, yeast and agar in plastic vials under 12 h day/night cycles at an incubation temperature of 25 °C unless otherwise stated. Glia-exclusive expression of transgenes was achieved via the bipartite GAL4/upstream activation sequence (UAS) system (reviewed in (Cauchi and van den Heuvel, 2006)) making use of the *Repo*-GAL4 driver (Awasaki and Ito, 2004). All transgenes have been previously characterised and their provenance is referenced in Supplementary Table 1. Combination of various genetic tools was performed according to standard genetic crossing schemes.

Adult viability

Adult viability studies were conducted by crossing the *Repo*-GAL4 driver stock to lines harbouring single or a combination of transgenes. Incubation occurred at temperatures of 25 °C or 29 °C. Following eclosion, adult flies were screened and counted at regular intervals. Viability was calculated as the percentage of the number of adult flies with the appropriate genotype divided by the expected number for the cross.

Larval mobility assays

Third instar larvae of the appropriate genotype were transferred to a 0.7% agar plate. Following acclimatisation, the number of forward body wall contractions exhibited by the organism in 30 s were counted. Each larva was assessed three times before an average was taken.

Flight assay

Flight performance was assessed in the Droso-Drome apparatus (Cacciottolo et al., 2019), which consists of a 1L glass bottle coated with an alcohol-based sticky fluid, and divided into 2 sections, a lower one (Sector A) and an upper one (Sector B), whose lengths are 5 cm and 15 cm, respectively. Flies first underwent a 'warm-up' by inducing negative geotaxis in an empty tube for 6 times. Organisms were then dropped into the Droso-Drome to induce flight. The number of flies that fell to sector A was next counted, divided by the total number of flies dropped and multiplied by 100 to generate the percentage of flies that were non-fliers.

Immunohistochemistry

Body wall muscles of wandering third instar larvae were dissected in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS and washed in PBS + 0.1% Triton X-100 (PBT). Tissues were then stained overnight at room temperature by mouse anti-Discs large antibody (1:1000; Developmental Studies Hybridoma Bank, University of Iowa, USA). On the following day, tissues were washed in PBT and stained overnight at room temperature with anti-mouse Alexa Fluor 488-conjugated secondary goat antibody (1:50). After a final wash in PBT, the samples were mounted in 90% glycerol with anti-fade. Imaging was performed with Optika B-600TiFL microscope (20× or 40× objectives) using brightfield and fluorescent light channels.

Analysis of muscle size and NMJ morphology

ImageJ software (NIH) was used to quantify both muscle and NMJ area. The former comprised of both ventral longitudinal muscles 6 and 7 derived from abdominal segments 2–4 whereas the latter constituted the postsynaptic region on the same muscles stained by the anti-Discs large antibody. Branch number was determined by counting the number of arborisations containing at least two boutons within a single NMJ. To determine, bouton numbers, all boutons were counted within a single NMJ.

Statistical analysis

Values are presented as mean ± S.E.M. One sample *t*-test was used to compare mean of experimental group with mean of control and the one-way ANOVA with Dunnett's *post hoc* test was used for multiple comparisons with the control (GraphPad Prism v9.3.0). Differences were deemed statistically significant if $p < 0.05$.

RESULTS

Loss of Smn function in glia has a negative impact on adult viability

In *Drosophila*, reduced levels of Smn in all tissues induces lethality (Chan et al., 2003; Chang et al., 2008). This outcome is also achieved when Smn is selectively reduced in muscles rather than neurons (Chang et al., 2008). Here, we asked whether a reduction of Smn levels specifically in glia, starting from early development, has an impact on the survival of the organism. To this end, we made use of the *Repo*-GAL4 driver to induce the expression of an RNAi transgenic construct targeting the full-length *Smn* transcript (*Smn RNAi*^{FL26B}) in glia of wild-type flies. Although this had minimal effect on fly viability (25 °C: 80.4% ± 6.5, 29 °C: 83.3 ± 7.1), we show that when the efficiency of knockdown was intensified by increasing Dicer-2 (Dcr-2) levels, a significant reduction in adult viability (32.6% ± 5.2) was achieved (Fig. 1). A boost in RNAi expression brought about by culturing flies at a temperature of 29 °C, known to induce maximal GAL4 activity, resulted in a further decrease in survival (1.1% ± 0.6). On using another RNAi transgene, targeting the 3′ end of the *Smn* mRNA transcript (*Smn RNAi*^{C24}), we also observed a significant drop in adult viability (25.1% ± 8.7) when flies were cultured at higher temperatures and, in the presence of extra levels of Dcr-2, total lethality was induced.

We further show that glial-selective expression of an RNAi transgene targeting the 5′ end of the *Smn* mRNA transcript (*Smn RNAi*^{N4}) caused lethality at restrictive culture conditions (Fig. 1). Supraphysiological levels of Smn in all tissues was found to have no effect on *Drosophila* survival (Grice and Liu, 2011), and we achieved a similar outcome when Smn was overexpressed specifically in glia. However, overexpression of the human version of Smn (hSMN), known to antagonise endogenous Smn function (Miguel-Aliaga et al., 2000; Borg and Cauchi, 2013), was also found to reduce fly viability (21.1% ± 6.4) at a culture temperature that permits enhanced transgenic expression (29 °C). In sum, these findings show that Smn is required in glia from early development, hence, reduced levels of Smn have negative consequences on *Drosophila* viability.

Gain rather than loss of TDP-43, FUS or C9orf72 function in glia leads to reduced adult viability

We wanted to investigate whether disruption of ALS-linked genes effects *Drosophila* survival in a similar manner to that observed on loss of Smn function in glia. We first show that, in contrast to Smn (above), reduced levels of TBPH (the *Drosophila* orthologue of TDP-43) in glia had no effect on fly viability, even when flies were subjected to conditions that permit enhanced RNAi-mediated knockdown (Fig. 2A). However, overexpression of TBPH selectively in glia induced a marked decrease in survival, the effect of which was more pronounced at culture conditions that permit

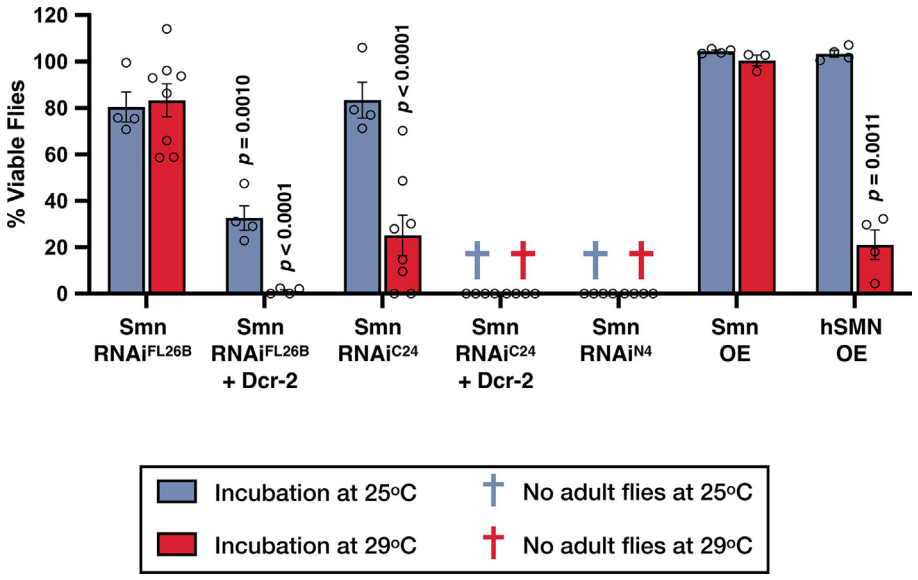


Fig. 1. Smn is indispensable for adult viability in glia. Bar chart showing percentage adult fly viability on expression of various transgenes targeting *Drosophila Smn* or its human counterpart SMN via the glia-specific *Repo*-GAL4 driver. Reduced Smn levels in glia led to a reduction in viability, the severity of which is dependent on RNAi efficiency. Glial-specific Smn overexpression (OE) had no effect on survival. However, expression of a transgene coding for human SMN induces reduced viability at a temperature of 29 °C, which is synonymous with higher GAL4 expression levels. Individual bars represent the mean adult viability ± S.E.M. normalised to the *Repo*-GAL4 driver control. Individual data points are superimposed on the bars. For each genotype, at least, three independent experiments were conducted ($n \geq 100$ per genotype) and viability was assayed at a temperature of 25 °C and 29 °C. Significance as tested by the one sample *t*-test is indicated by the exact *p*-value.

higher transgenic expression (25 °C: 59.7% ± 2.6, 29 °C: 30.1% ± 2.4). Again, the situation is the opposite of that observed for Smn (above). Expression of an epitope-tagged version of TBPH (*Flag.TBPH*) was found to induce complete lethality (Fig. 2A), suggesting that an enhanced phenotype can be brought about by a modification that interferes with the structure of the transgenic protein. Expression of human TDP-43 in glia was also found to depress survival at conditions that permit enhanced expression (29 °C) and/or upon expression of an epitope-tagged version (*Flag.TDP-43*). Furthermore, glial-selective expression of the major TDP-43 C-terminal fragment found in cytosolic aggregates of ALS patients (*TDP-43^{CTF}*), was found to induce complete lethality at less permissive culture conditions (Fig. 2A).

Next, we wanted to investigate whether similar to TBPH/TDP-43, glial-selective gain rather than loss of function of FUS or its *Drosophila* orthologue caz has a negative influence on viability. We find that similar to TBPH, reduced levels of caz in glia had no effect on fly survival (Fig. 2B). Upregulation of caz had minimal effect on viability at 29 °C (90% ± 6.1). However, expression of either wild-type

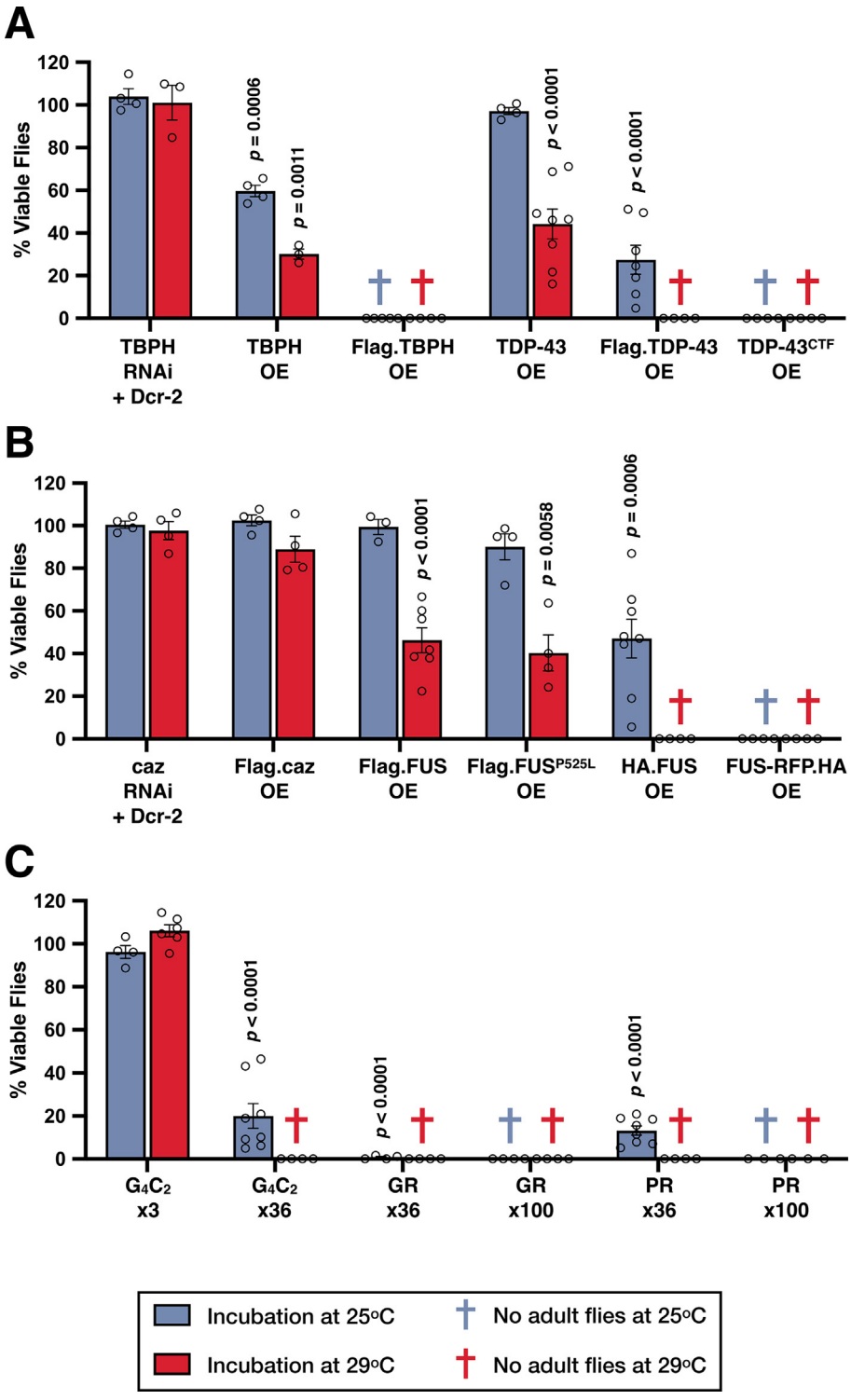


Fig. 2. Gain of TDP-43, FUS or C9orf72 function in glia is detrimental for survival. **(A)** Bar chart showing percentage adult fly viability on expression of various transgenes targeting TBPH or its human counterpart TDP-43 in glial tissues. Reduction in TBPH had no effect on fly viability. Gain of function through TBPH/TDP-43 overexpression (OE) had a negative impact on survival, the severity of which is dependent on expression levels, epitope-tagging and/or expression of truncated versions. **(B)** Bar chart showing percentage adult fly viability on expression of various transgenes targeting caz or its human counterpart FUS in glia. Downregulation or upregulation of caz had no or minimal effect on survival. Expression of FUS depressed survival, the degree of which depends on expression levels and/or epitope-tagging. **(C)** Bar chart showing percentage adult viability on expression of C9orf72-associated hexanucleotide repeats or DPRs in glia. Only pathogenic repeat lengths (G_4C_2 X36) induced a marked decrease in viability at 25 °C and lethality at higher expression levels (29 °C). Expression of glycine-arginine (GR) or proline-arginine (PR) DPRs also effected survival, with phenotypes that are more pronounced at high expression levels and/or when lengthier repeats are expressed. GR DPRs were more toxic in glia than PR DPRs. In **(A–C)**, individual bars represent the mean adult viability ± S.E.M. normalised to the *Repo*-GAL4 driver control. Individual data points are superimposed on the bars. For each genotype, at least, three independent experiments were conducted ($n \geq 100$ per genotype) and viability was assayed at a temperature of 25 °C and 29 °C. Significance as tested by the one sample *t*-test is indicated by the exact *p*-value.

human FUS or a version with a pathogenic mutation in the C-terminus (FUS^{P525L}) induced a significant drop in viability when the transgenes were expressed at a temperature of 29 °C which permits maximal transgenic expression (Fig. 2B). Expression of an HA-tagged FUS transgene had a greater impact on survival, inducing reduced viability at 25 °C and complete lethality at 29 °C. Furthermore, expression of a FUS transgene tagged with both RFP and HA also induced lethality, however, at less permissive culture conditions (Fig. 2B). Turning to C9orf72, which has no orthologue in *Drosophila*, we show that whereas transgenic expression of a non-pathogenic hexanucleotide repeat length (G_4C_2 ×3) in glial cells had no effect on survival, expression of 36 repeats (G_4C_2 ×36), previously shown to be toxic in neurons (Mizielinska et al., 2014), caused a marked reduction in viability at 25 °C (20% ± 5.8) and complete lethality at 29 °C (Fig. 2C). It is noteworthy that a similar or a more drastic outcome was observed when assessing animals expressing two arginine-containing DPR proteins, including glycine-arginine (GR ×36, GR ×100) or proline-arginine (PR ×36, PR ×100) at both restrictive (25 °C) and non-restrictive (29 °C) temperatures (Fig. 2C). These results underscore that in contrast to Smn, gain

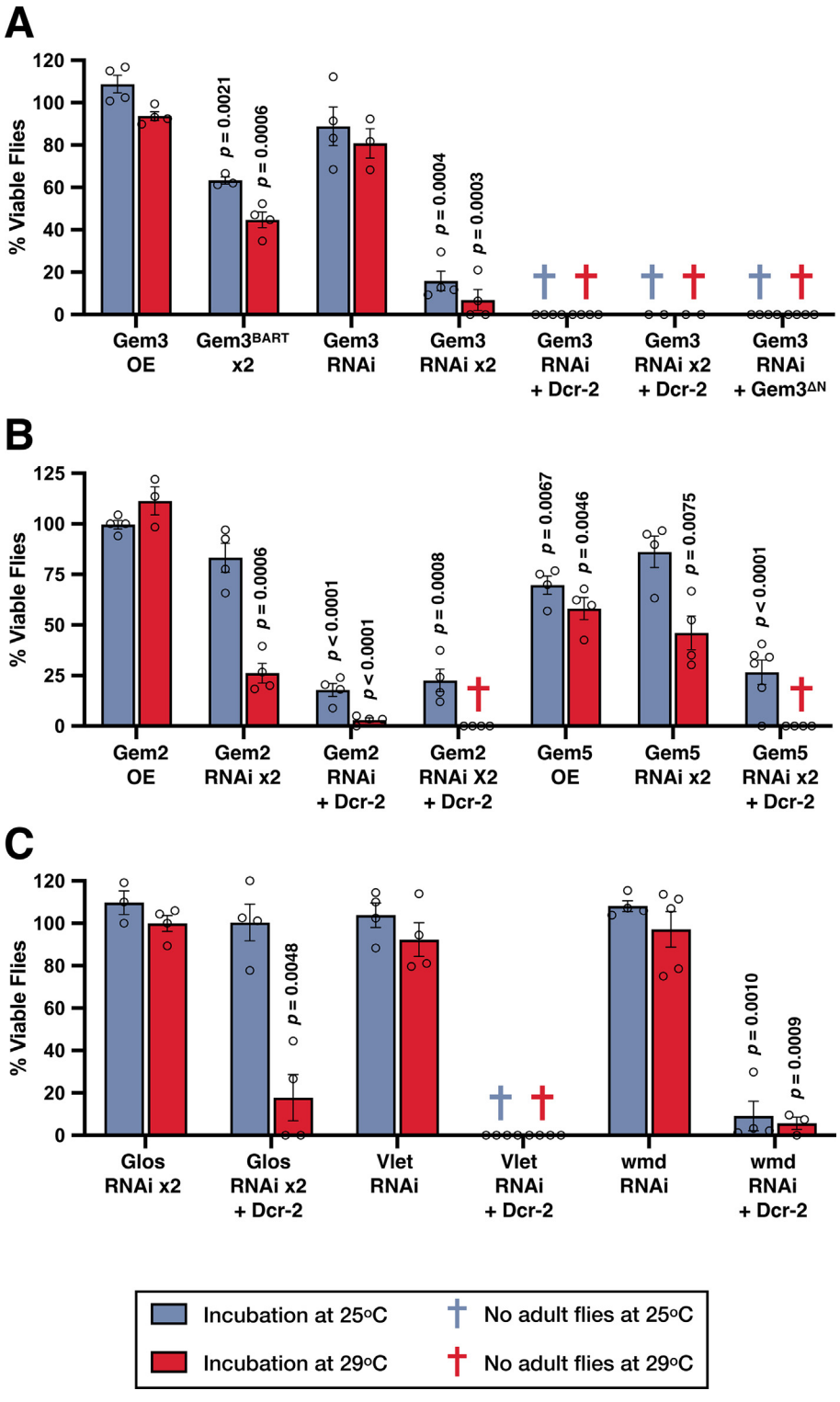


Fig. 3. Glial-selective disruption of Gemins 2–5, Gemin8 or Unrip has a deleterious effect on survival. **(A)** Bar chart showing percentage adult fly viability on expression of various transgenes targeting *Drosophila* Gem3 in glia. Gem3 upregulation did not impact viability. However, loss of Gem3 function brought about by RNAi or expression of Gem3 truncated alleles induced reduced survival or complete lethality. **(B)** Bar chart showing percentage adult fly viability on expression of transgenes targeting *Drosophila* Gem2 and Gem5 in glia. Although Gem2 overexpression was uneventful, RNAi-mediated knockdown induced a drop in viability or complete lethality depending on RNAi efficiency. Gem5 upregulation caused a moderate decrease in survival. Downregulation had a deleterious effect on viability, whose severity is also dependent on RNAi efficiency. **(C)** Bar chart showing percentage adult fly viability upon glial-selective expression of transgenes targeting *Drosophila* Glos, Vlet and wmd, the *Drosophila* orthologues of Gem4, Gem8 and Unrip. An enhanced reduction in the levels of Glos, Vlet or wmd was detrimental to organismal viability. In **(A–C)**, individual bars represent the mean adult viability ± S.E.M. normalised to the *Repo*-GAL4 driver control. Individual data points are superimposed on the bars. For each genotype, at least, three independent experiments were conducted ($n \geq 100$ per genotype) and viability was assayed at a temperature of 25 °C and 29 °C. Significance as tested by the one sample *t*-test is indicated by the exact *p*-value.

rather than loss of TBPH/TDP-43, FUS or C9orf72 function in glia is required to induce a negative impact on survival.

Adult viability is negatively affected on disruption of Smn complex components in glia

We questioned whether the Smn complex is required in its entirety for glia to function properly. Hence, we selectively disrupted *Drosophila* Smn complex members in glia, focusing on those that are coded by essential genes (Cauchi et al., 2008; Borg and Cauchi, 2013; Borg et al., 2016; Lanfranco et al., 2017a). We find that upregulation of Gemin3 (Gem3) in glia had no effect on adult viability (Fig. 3A). However, a significant reduction in survival (25 °C: 63.3% ± 1.7, 29 °C: 44.8% ± 3.7) can be achieved on loss of Gem3 function induced by expression of a double dose of $Gem3^{BART}$, a weak version of the truncated $Gem3^{\Delta N}$ mutant (Borg et al., 2015). Knockdown of Gem3 also resulted in a dramatic dip in adult viability when a double dose of the RNAi transgene was expressed (25 °C: 15.9% ± 4.6, 29 °C: 6.9% ± 5). Enhanced loss of Gem3 function in glia through an increase in RNAi efficiency brought about by expression of Dcr-2 led to complete lethality (Fig. 3A). A similar outcome was achieved when expression of a Gem3 RNAi transgene was combined with the strong $Gem3^{\Delta N}$ mutant (Fig. 3A).

Whereas glial-specific overexpression of Gemin 2 (Gem2) had no effect on survival, downregulation brought about by the expression of two RNAi transgenes led to a marked reduction in viability especially when culture temperature was increased, hence allowing for maximal expression (25 °C: 83.3% ± 7.1, 29 °C: 26.2% ± 4.8) (Fig. 3B). A similar outcome was achieved at a less permissive temperature (25 °C) when RNAi efficiency was improved through expression of Dcr-2 combined with either one (17.9% ± 3.2) or two Gem2 RNAi transgenes (22.6% ± 5.6). In case of the latter, complete lethality was the outcome when flies are cultured at a permissive temperature (Fig. 3B). Turning to Gemin5 (Gem5), its overexpression specifically in glia had a moderate yet statistically significant impact on adult viability (25 °C: 69.7% ± 4.5, 29 °C: 58.1% ± 5.5). Glial-selective reduction of Gem5, induced through the expression of two RNAi transgenes, also led to a substantial decrease in fly viability at a culture temperature of 29 °C (46.1% ± 8.3). When RNAi efficiency was enhanced by extra levels of Dcr-2, a significant reduction in survival (26.6% ± 6.1) and complete lethality were achieved at 25 °C and 29 °C, respectively. Finally, we show that a dramatic drop in survival or complete lethality can also be attained by glia-selective enhanced expression of RNAi transgenes targeting Glos, Vlet and wmd, the *Drosophila* orthologues of Gemin4, Gemin8 and Unrip, respectively (Fig. 3C). Overall, these results suggest that in addition to Smn, Smn complex members Gemin2/3/4/5/8 and Unrip are essential in glia and their loss leads to detrimental effects on *Drosophila* survival.

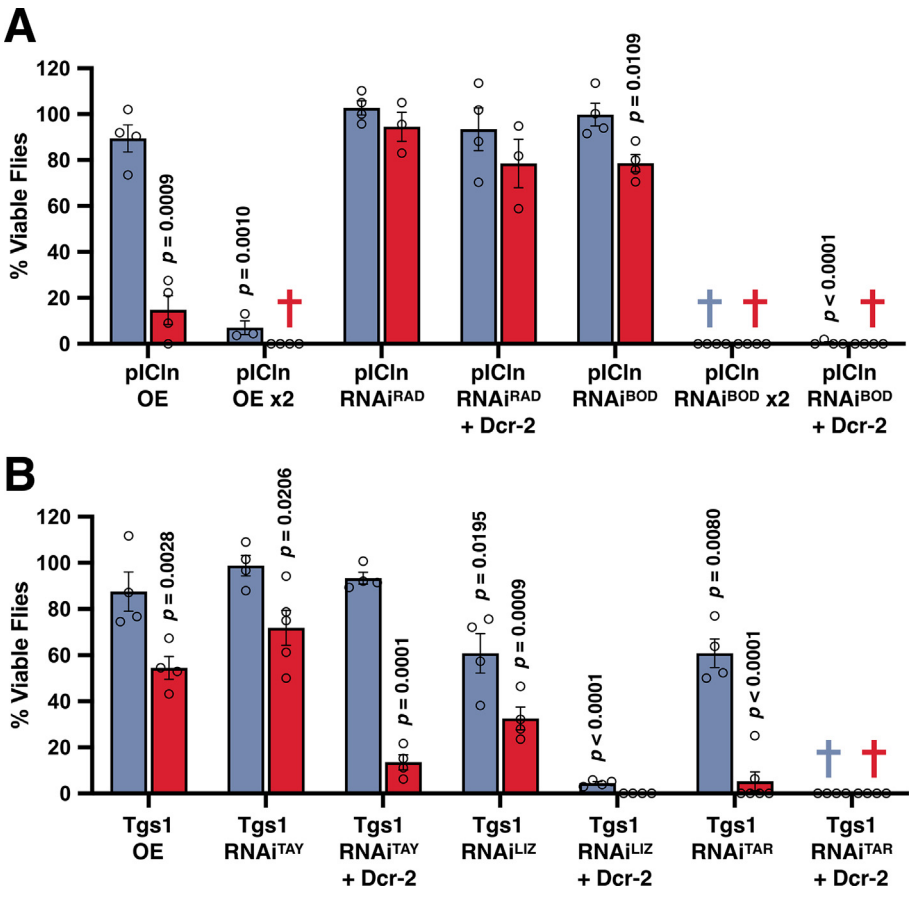


Fig. 4. Viability is negatively affected by glial-selective perturbation of pICln and Tgs1 levels. **(A)** Bar chart showing percentage adult fly viability on expression of various transgenes targeting *Drosophila* pICln in glia. pICln upregulation had a negative influence on survival, the degree of which is dependent on expression levels. Loss of pICln function brought about by use of the *pICln* $RNAi^{BOD}$ transgene caused complete or nearly complete lethality when a double dose was applied or in the presence of boosted Dcr-2 levels. **(B)** Bar chart showing percentage adult fly viability on expression of various transgenes targeting Tgs1. Tgs1 overexpression induced reduced viability at 29 °C. Use of various RNAi transgenes targeting Tgs1 all had a deleterious effect on fly viability, the severity of which was dependent on use of high culture temperatures and extra Dcr-2 levels. In **(A, B)**, individual bars represent the mean adult viability ± S.E.M. normalised to the *Repo*-GAL4 driver control. Individual data points are superimposed on the bars. For each genotype, at least, three independent experiments were conducted ($n \geq 100$ per genotype) and viability was assayed at a temperature of 25 °C and 29 °C. Significance as tested by the one sample *t*-test is indicated by the exact *p*-value.

Perturbation of snRNP biogenesis factors pICln and Tgs1 specifically in glia causes viability defects

We wished to gain insights on the role of the Smn complex within glia, specifically asking whether its cardinal function in snRNP biogenesis is required in glia during development. With this in mind, we first disrupted pICln, a factor acting in the early phase of snRNP assembly. Overexpression of pICln in glia had a negative influence on viability, the degree of which is dependent on expression levels. Hence, a single pICln transgene only induced a marked drop in viability at 29 °C (14.7% ± 6.3) whereas expression of two pICln transgenes was able to cause reduced survival at a less permissive culture temperature (25 °C: 7% ± 3) and complete lethality at 29 °C, a more permissive culture temperature (Fig. 4A). Although adult viability is refractory to glial-selective reductions in pICln levels when using a low-expressing RNAi transgene (*pICln RNAi*^{RAD}), use of high-expressing RNAi transgene (*pICln RNAi*^{BOD}) and an increase in RNAi efficiency achieved by expressing either a double dose or extra levels of Dcr-2, resulted in lethality (Fig. 4A). We next asked whether similar results can be obtained on perturbation of Tgs1, a factor involved in the final phases of snRNP biogenesis. We show that Tgs1 upregulation depressed survival at more permissive culture conditions (25 °C: 87.6% ± 8.5, 29 °C: 54.5% ± 5). Reductions in Tgs1 levels via either of three RNAi transgenes also had a deleterious effect on adult viability, the severity of which was proportional to RNAi efficiency (Fig. 4B). These findings suggest that glia are dependent on snRNP biogenesis for their function during *Drosophila* development.

Glial-selective loss of Smn function has no effect on neuromuscular performance

We next wished to investigate whether loss of Smn function has a negative impact on motoric performance in addition to adult fly viability. In this regard, we first assessed the mobility of third instar larvae of flies in which the specific loss of Smn function in glia induces adult lethality. Interestingly, we find that RNAi-mediated Smn knockdown boosted by Dcr-2 had no effect on the contraction rate of larvae compared to the control (Fig. 5A). Similarly, we show that enhanced knockdown of Smn complex member Gem3 in glia did not cause a reduction in the mobility of larvae prior to their death (Fig. 5A). Notably, the same outcome was also observed when we knocked down either of the snRNP biogenesis factors pICln or Tgs1 in glia. Thus, larvae had no obvious mobility deficits although they perished before reaching the adult stage (Fig. 5A). Conversely, whereas upregulation of TBPH induced lethality before the third instar larval stage, overexpression of TDP-43^{CTF} was found to cause a profound reduction in the larval contraction rate (Fig. 5B). The same consequence was observed on overexpression of human FUS in glia (Fig. 5B). Expression of ×36 GR DPRs also induced a significant decline in larval mobility whereas ×100 GR DPRs led to death before the third instar larval stage

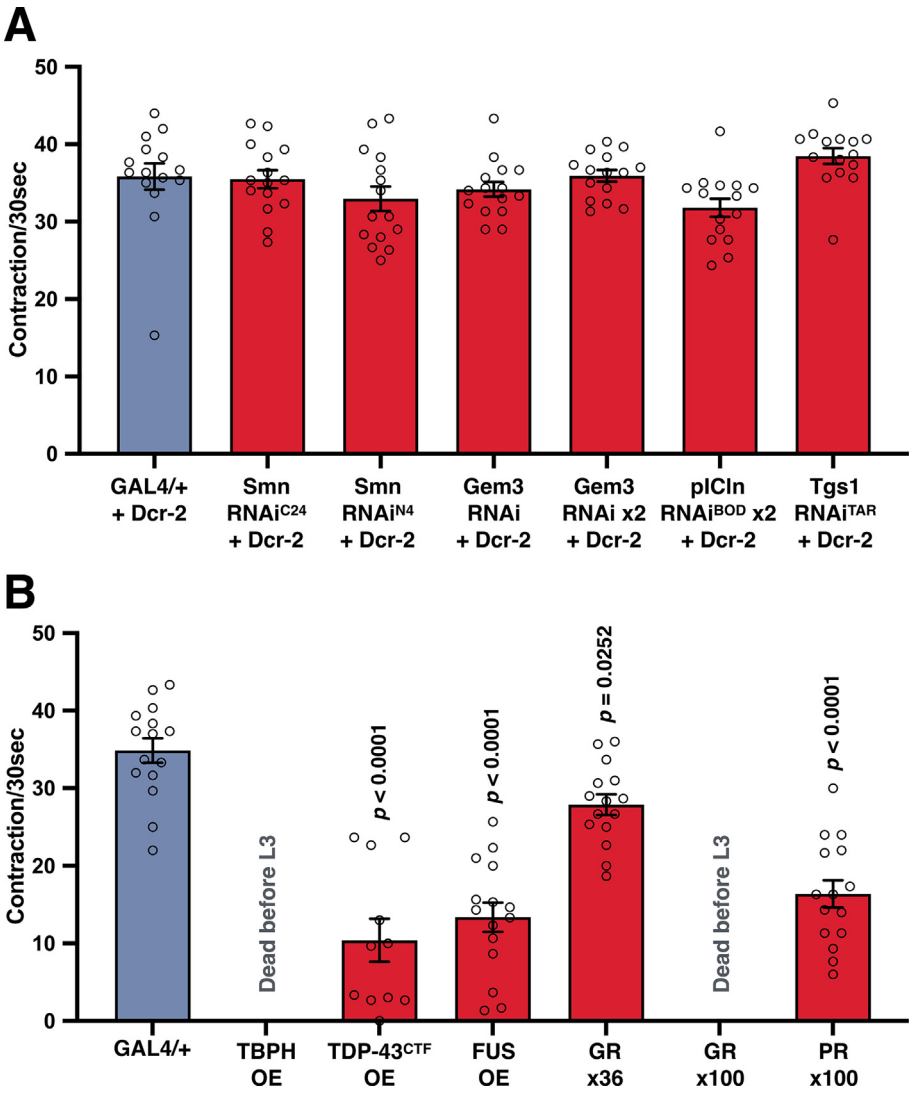


Fig. 5. Mobility in larvae with various genetic manipulations selectively directed to glia. **(A)** Reduced levels of Smn, Gem3, pICln or Tgs1 had no effect on larval contraction rate. **(B)** Expression of TDP-43, FUS or DPRs induced mobility defects in larvae. In **(A, B)**, individual bars represent the mean ± S.E.M. and $n = 15$ per genotype. Individual data points are superimposed on the bars. Significance as tested by one-way ANOVA with Dunnett's *post hoc* test is indicated by the exact *p*-value.

precluding us from assessing these flies. Flies with glial-selective expression of x100 PR DPRs perish before the adult stage, and larvae were likewise found to be extremely sluggish in contrast to the control (Fig. 5B). Turning to adult fly escapers, we show that in contrast to flies with glia-selective expression of TDP-43, FUS or x36 PR DPRs which all show a degree of flight defects at day 5 post-eclosion, enhanced knockdown of Smn or its associate Gem3 had no effect on flight performance (Fig. 6). Overall, these results underscore that Smn or the Smn complex is not essential for motor performance in glia.

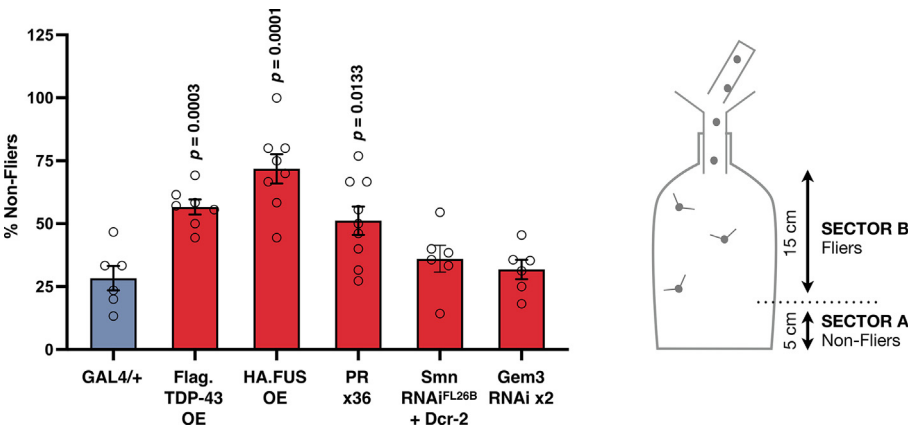


Fig. 6. Disruption of Smn or Gem3 has no effect on flight performance in contrast to gain of TDP-43, FUS or C9orf72 function. Bar chart showing the percentage of flightless flies or flies that fall straight to Sector A of the Droso-Drome apparatus (*Right*). Flies assessed were aged to day 5 post-eclosion. Adult escapers with glial-selective overexpression of TDP-43, FUS or ×36 PR DPRs had flight defects. In contrast, adult flies with enhanced reduction of either Smn or Gem3 in glia were good fliers. Individual bars represent the mean ± S.E.M. of at least four independent experiments and $n \geq 120$ per genotype. Individual data points are superimposed on the bars. Significance as tested by one-way ANOVA with Dunnett's *post hoc* test is indicated by the exact *p*-value.

Reduced levels of Smn in glia do not induce profound NMJ defects or muscle atrophy

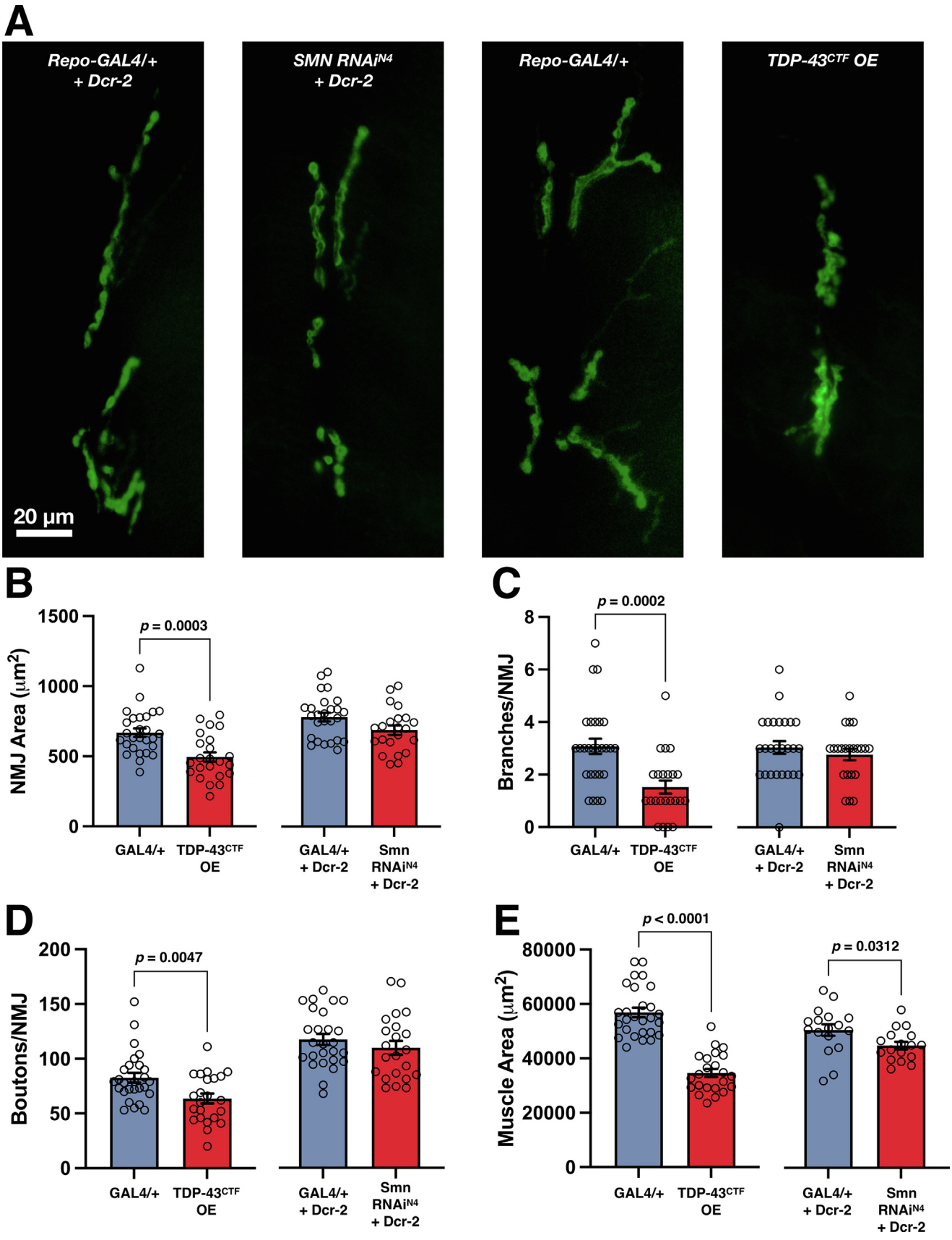
Finally, we sought to determine whether reduced levels of Smn in glia have any effect on motor neurons and muscle tissue. To this end, we assessed the morphology of the neuromuscular junction (NMJ) of motor neurons innervating ventral longitudinal muscles 6 and 7 within the abdominal segments 2–3 of larvae with glia-specific enhanced reduction of Smn levels. On visual inspection, no gross abnormalities were observed (Fig. 7A). Indeed, following quantitation, we found that Smn knockdown had no effect on NMJ size, number of NMJ branches or bouton numbers (Fig. 7B–D). This is in contrast to glial-selective overexpression of TDP-43^{CTF}, which was found to induce small NMJs on visual observation (Fig. 7A). This phenotype was confirmed on quantitation, hence, a statistically significant difference was noted for all the measured NMJ morphology parameters as compared to the control (Fig. 7B–D). Furthermore, by analysing the combined muscle area of ventral longitudinal muscle 6 and 7, we note that gain of TDP-43 function caused a marked decrease in muscle area whereas loss of Smn function only induced slight differences when compared to the control (Fig. 7E). In sum, these observations indicate that Smn is not required in glia for maintaining muscle size or neuromuscular junctions.

DISCUSSION

In this study, we made use of the *Drosophila* model system to determine the requirements of Smn in glia. We find that survival rather than motor behaviour is profoundly affected when Smn is reduced selectively in glia. A similar outcome was observed on disruption of the Smn complex and key snRNP biogenesis factors, suggesting that the role of Smn in snRNP biogenesis as part of the Smn complex is required for glia to support the survival of the organism during its early developmental stages. Nonetheless, an interference with this function has no effect on the ability of glia to influence neuromuscular function. This is in contrast to disruption of ALS-linked TDP-43, FUS and C9orf72, which in addition to affecting *Drosophila* survival also induce motor impairments and, in case of TDP-43, leads to neuromuscular tissue morphology defects.

To our knowledge, the role of Smn or the Smn complex in the glia of *Drosophila* has not been investigated previously. The fly model was however crucial to demonstrate a requirement for ALS-linked TBPH/TDP-43 in glia for survival, locomotor activity, sleep, motor neuron axon wrapping and synapse formation (Diaper et al., 2013; Estes et al., 2013; Romano et al., 2015). Here, we also show that similar to TDP-43, gain of FUS or C9orf72 function directed to glia also impairs survival and motor behaviour. It is noteworthy that loss of TBPH/TDP-43 or caz/FUS function in glia

Fig. 7. Reduced levels of Smn in glia do not induce dramatic neuromuscular morphology defects in contrast to overexpression of TDP-43^{CTF}. **(A)** Representative images of NMJs innervating longitudinal muscles 6 and 7 in third instar larvae stained with post-synaptic anti-DLG antibody. Visual inspection revealed that compared to their respective control, glia-directed expression of TDP-43^{CTF} but not *Smn RNAi*^{N4} induced dramatically undergrown NMJs. These observations were confirmed upon quantification of NMJ area **(B)**, number of branches per NMJ **(C)** and number of boutons within a single NMJ **(D)**. **(E)** Muscle area is profoundly reduced in TDP-43^{CTF} overexpressors whereas only a small difference was observed in flies with a reduction in Smn levels directed to glia. In **(B–E)** data presented are the mean ± S.E.M. and $n \geq 17$ per genotype. Individual data points are superimposed on the bars. Significance as tested by the unpaired *t*-test is indicated by the exact *p*-value.


A
Repo-GAL4/+
+ Dcr-2
SMN RNAi^N4
+ Dcr-2
Repo-GAL4/+
TDP-43^CTF OE
20 µm
B
NMJ Area (µm²)
p = 0.0003
GAL4/+
TDP-43^CTF OE
GAL4/+
+ Dcr-2
Smn RNAi^N4
+ Dcr-2
C
Branches/NMJ
p = 0.0002
D
Boutons/NMJ
p = 0.0047
E
Muscle Area (µm²)
p < 0.0001
p = 0.0312

has no negative consequences on *Drosophila* survival during development, which is in contrast to that observed on reduction of Smn or Smn complex members Gemin2/3/4/5/8. Interestingly, this is reflective of the pathoaetiology of SMA and ALS, where the latter mostly results from dominant gain of function mutations whereas the former is the result of recessive loss of function mutations in SMN.

In line with previous reports, our results underscore that SMN is required in glia for the survival of the organism. Hence, restoration of SMN in both glia and motor neurons or solely in glia was found to increase the lifespan of SMA mice (McGovern et al., 2015; Rindt et al., 2015). The opposite is also true since we show that a decrease of Smn in glia reduces the survival of flies. We extend a glial-specific requirement for survival during development for several Smn complex members in addition to key snRNP biogenesis factors pICln and Tgs1. Thus, it is reasonable to speculate that the role of Smn in snRNP assembly as a core member of the Smn complex is required within glia for them to support the survival of the organism. A recent study has since confirmed a requirement for Gemin3 in oligodendrocytes for normal lifespan in mice (Simankova et al., 2021). Nonetheless, whereas others showed improvements in motor function when SMN is restored in the glia of SMA mice models (Rindt et al., 2015; Hunter et al., 2016), we find that a glial-selective reduction in Smn, Gem3, pICln or Tgs1 had no negative impact on motoric performance. Differences between mice and fly models with regards to glial populations and/or functions may be one of the reasons for this discrepancy. Moreover, the type of genetic manipulation is different between studies. Whereas we assessed organisms with wild-type tissues except glia, mouse studies did the reverse, hence, assessing organisms with loss of SMN function in all tissues except glia (Rindt et al., 2015; Hunter et al., 2016).

Our study joins others in underscoring the importance of glia in the pathophysiology of SMA. Hence, it is now believed that similar to ALS, SMA is a non-cell-autonomous disease with other cells including glia impacting significantly on the disease process. Our work indicates that the role of Smn in snRNP assembly as part of the Smn complex is essential for the normal function of glia. Like motor neurons (Lanfranco et al., 2017b), glia may therefore be susceptible to defects in RNA metabolism that impair their ability to provide the necessary support to neurons. Further investigations that reveal the molecular events downstream of Smn complex disruption or perturbation in snRNP biogenesis in glia are warranted. Importantly, our work and others highlight that SMA therapy should target glia in addition to motor neurons since this is predicted to result in better outcomes in patients.

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APPENDIX A. SUPPLEMENTARY DATA

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