Motor defects in a *Drosophila* model for spinal muscular atrophy result from SMN depletion during early neurogenesis

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Abstract

Spinal muscular atrophy (SMA) is the most common autosomal recessive neurodegenerative disease, and is characterised by spinal motor neuron loss, impaired motor function and, often, premature death. Mutations and deletions in the widely expressed *survival motor neuron 1* (*SMN1*) gene cause SMA; however, the mechanisms underlying the selectivity of motor neuron degeneration are not well understood. Although SMA is degenerative in nature, SMN function during embryonic and early postnatal development appears to be essential for motor neuron survival in animal models and humans. Notwithstanding, how developmental defects contribute to the subversion of postnatal and adult motor function remains elusive. Here, in a *Drosophila* SMA model, we show that neurodevelopmental defects precede gross locomotor dysfunction in larvae. Furthermore, to specifically address the relevance of SMN during neurogenesis and in neurogenic cell types, we show that SMN knockdown using neuroblast-specific and pan-neuronal drivers, but not differentiated neuron or glial cell drivers, impairs adult motor function. Using targeted knockdown, we further restricted SMN manipulation in neuroblasts to a defined time window. Our aim was to express specifically in the neuronal progenitor cell types that have not formed synapses, and thus a time that precedes neuromuscular junction formation and maturation. By restoring SMN levels in these distinct neuronal population, we partially rescue the larval locomotor defects of *Smn* mutants. Finally, combinatorial SMN knockdown in immature and mature neurons synergistically enhances the locomotor and survival phenotypes. Our in-vivo study is the first to directly rescue the motor defects of an SMA model by expressing *Smn* in an identifiable population of *Drosophila* neuroblasts and developing neurons, highlighting that neuronal sensitivity to SMN loss may arise before synapse establishment and nerve cell maturation.

Author summary

Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality and leads to the degeneration of the nerves that control muscle function. Loss-of-function
mutations in the widely expressed *survival motor neuron 1* (*SMN1*) gene cause SMA, but how low levels of SMN protein cause the neuronal dysfunction is not known. Although SMA is a disease of nerve degeneration, SMN function during nerve cell development may be important, particularly in severe forms of SMA. Nevertheless, how the defects during development and throughout early life contribute to the disease is not well understood. We have previously demonstrated that SMN protein becomes enriched in neuroblasts, which are the cells that divide to produce neurons. In the present study, motor defects observed in our fly model for SMA could be rescued by restoring SMN in neuroblasts alone. In addition, we show that knocking down SMN in healthy flies within the same cell type causes impaired motor function. The present study shows that the manipulation of SMN in a developmentally important cell type can cause motor defects, indicating that a period of abnormal neurodevelopment may contribute to SMA.

**Introduction**

Survival motor neuron (SMN) is an essential protein that functions in the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs), which subsequently mediate pre-mRNA splicing [1]. Loss-of-function mutations in the *SMN1* gene cause the disease spinal muscular atrophy (SMA), which is characterised by the selective loss of alpha motor neurons of the spinal cord, muscle wasting and, in most severe cases, premature death in infancy [2].

Since the identification of the disease-associated gene *SMN1* in 1995 [2], the drive to uncover the mechanisms underlying SMA pathogenesis has been complicated by the pleiotropic nature of the SMN locus [3], coupled with the varied levels of SMN protein in human and animal models [4–6]. There has been considerable debate about how aberrations in both the canonical and non-canonical motor neuron-specific functions of SMN may lead to the observed motor neuron selectivity [7]. SMN has been shown to play a fundamental role in snRNP and messenger ribonucleoprotein (mRNP) biogenesis [8], whilst also being involved in mRNA trafficking and local translation, cytoskeletal dynamics, endocytosis and ubiquitin homeostasis (reviewed in [3,9]). In addition, the nature of SMA pathology, and of the animal models engineered to study the disease, are greatly affected by the systemic, temporal and spatial levels of SMN protein [5,6]. In humans, in addition to *SMN1*, SMN is also encoded by a second paralogous gene called *SMN2*, which, owing to a mutation affecting exon 7 splicing, generates comparatively low levels of full-length SMN protein [10,11]. Due to the fact that *SMN2* copy number can also vary between individuals, there is a broad spectrum of disease severity that, at the population level, correlates with the amount of *SMN2*-derived wild-type SMN protein [4]. As SMN levels decrease, disease severity increases, the motor defects become more pronounced, and many more cell and tissue types present with phenotypes caused by loss of the protein [12,13].

Classed as a ubiquitous protein, SMN localises to the cytoplasm and nucleus, and can be observed in many RNP-enriched subcellular foci, such as Gems [14], nucleoli [15], U bodies [16] and Cajal bodies [14]. Cells do not necessarily require organised Gems, U Bodies and Cajal bodies to survive; however, evidence shows that these cellular foci promote the efficient clustering of the RNA processing factors required in embryonic, dividing and metabolically active cells [17]. SMN protein level and associated snRNP assembly are highest during embryonic development, and are substantially downregulated postnatally [18] and as cells differentiate and mature [19–21]. Furthermore, severe SMN loss can lead to developmental defects,
with a hierarchy of cell types, many of which are uncharacterised, having differing sensitivities to a reduction in the level of the protein [6,12].

Undoubtedly, the alpha motor neurons are particularly sensitive to SMN reduction. Notwithstanding, it is not known how this selectivity manifests in its entirety (i.e., whether it is a result of aberrations in set-up or degeneration, or if it is through a non-cell autonomous mechanism). Previous research has shown that the selective loss of SMN in motoneuronal progenitors is sufficient to cause SMA like phenotypes [22]. Furthermore, restoration of SMN in mature motoneurons only rescued the SMA phenotype partially [23,24], whereas motor neuron-specific SMN reduction in wild type mice fails to recapitulate the entirety of the disease phenotypes, highlighting the importance of neuronal development. Motor neuron loss is also a relatively late feature in SMN patients and mammalian models [25, 26], although patients with type 1 SMA present with neuromuscular junction (NMJ) maturation defects during fetal development [27]. Importantly, when performing rescue studies using mouse and Drosophila SMA models, early stage ubiquitous restoration of SMN results in the greatest improvement in motor function and animal survival [26,28,29]. This is supported by evidence from patient clinical trials [30] and early versus later treatment of SMA mice [6,31–37]. To complement these findings, SMN reduction in young adult mice caused more modest phenotypes when compared with mice in which SMN was knocked down at an earlier developmental time point [6, 26,28]. Furthermore, Drosophila studies using Smn mutant models have reported severe growth defects and considerable developmental retardation, in addition to motor and NMJ dysfunction [5,21,38–41]. In mouse embryos, although no overt developmental outgrowth defects have been observed [42], defective radial outgrowth and poor Schwann ensheathment led to some axons degenerating postnatally [43]. Altogether, this research highlights that the cause of SMA may not be solely through classical neurodegenerative processes, but via a combinatorial multi-cell type mechanism that may be sensitised by neurodevelopmental abnormalities. An understanding of the precise nature of the developmental requirements of SMN, and how perturbations in SMN protein level leads to defects that manifest in progenitor and non-differentiated neuronal cell types, is important for SMA treatment.

The aim of the present study was to understand how manipulation of SMN protein level during specific periods of neurogenesis can cause and modify the phenotypes present in Drosophila models for SMA. The aim was to restrict SMN manipulation to the neuronal progenitor cell types that have not yet formed synapses, and to a period that precedes NMJ maturation. To achieve this, knockdown and rescue studies were used during the waves of proliferation and differentiation in the larval and pupal central nervous system (CNS). The classical GAL4 and the more targeted GAL80 repression systems were used to allow for spatiotemporal transgene expression [44]. The reduction of SMN in neuroblasts and undifferentiated neurons, but not subsequently in differentiated subpopulations of neurons, caused motor defects. In the reciprocal experiment, neurodevelopmental and motor phenotypes are partially rescued by expressing SMN in neuroblasts and immature neurons. Finally, combinatorial SMN knockdown was carried out in immature and mature neurons, which synergistically enhanced the locomotor and survival phenotypes in the present model. This in-vivo study contributes to the understanding of how developmental abnormalities can contribute to the motor defects synonymous with the pathology of SMA. Furthermore, by selectively manipulating SMN in an identifiable population of neuroblasts and developing neurons, we highlight the importance of SMN in Drosophila neurodevelopment.
Results

Developmental arrest precedes larval locomotion dysfunction in Smn mutants

During *Drosophila* embryonic neurogenesis, the nervous system required for larval life is generated [45–47]. Larvae then develop through three instar stages (L1–L3) before they pupate and become adults. During larval life, a second wave of neurogenesis occurs, and about 90% of adult neurons are created [48]. Loss-of-function and null *Drosophila* Smn mutants survive until larval stage on a maternal contribution of SMN protein [40]. This shortened lifespan is observed in the micro-deletion Smn<sup>x7</sup>, which removes the Smn coding region and is therefore classified as a null [39], and the point mutant Smn<sup>A</sup>, which acts as a loss-of-function [40]. In the present study, Smn<sup>A</sup>/Smn<sup>x7</sup> trans-heterozygotes (herein referred to as Smn mutants) were used to reduce the influence of genetic background. Although no embryonic defects have been described previously [49], significantly fewer Smn mutant larvae hatching than expected were observed at 25˚C and 20˚C (Fig 1B). Both 25˚C and 20˚C are the commonly used rearing temperatures that confer approximately an 11-day and 14-day lifecycle in the fly, respectively. For these experiments, the use of the life cycle lengths allowed the timeline of the developmental and motor phenotypes observed in the Smn mutants to be plotted more accurately. Homozygotes of both Smn<sup>A</sup> and Smn<sup>x7</sup> can live for several days in a developmentally immature larval state [5,21,39,40], and die as larvae when maternal SMN becomes depleted. The remaining hatched Smn mutants were tested and a comparable survival timeline was found, with larvae dying at a median of 3 and 4 days when kept at 25˚C and 20˚C, respectively (Fig 1C).

At the end of L1 (24 h after hatching, approximately 48 h after egg laying), most neuroblasts exit quiescence and start to divide [48]. As neuroblasts exit quiescence, they become enriched with SMN [21], and clonal knockout of Smn in neuroblasts has been shown to limit cell division and alter the clonal structure of the daughter cells generated from the SMN-deficient stem cells [21]. To determine the timing of proliferation defects in relation to Smn mutant larval lifespan, the number of nuclei stained positively for s-phase incorporated 5-ethynyl-2’-deoxyuridine (EdU) over a 72-h period were scored. Except for a small population of continuously dividing neuroblasts (some lateral and mushroom body neuroblasts bypass quiescence at the end of embryogenesis), low levels of EdU incorporation were observed in Smn mutants. A significant difference in EdU foci can be observed from 24 h when reared at both 25˚C and 20˚C (Fig 1D and 1E). This difference widened at 48 and 72 h after hatching (Fig 1E). After 72 h as larvae, the CNS of the remaining Smn mutant larvae remained significantly under-developed compared with wild-type larvae, when cultured at 25˚C and 20˚C (Fig 1D), highlighting that neuroblasts fail to significantly proliferate during the attenuated larval survival period. These data suggest that neuroblasts either fail to reactivate or that they generate only a nominal number of immature neurons and glia.

As SMA is a disorder of the motor system, larval locomotor dysfunction is often used as a proxy for motor abnormalities. The number of larval peristaltic muscle contractions that drive larval movement was next quantified. This is a method that has previously been used in the analysis of *Drosophila* models of neuropathy and SMA [50,51]. These contractions involve the rhythmic and sequential contraction of body-wall muscles, and are controlled by an intricate circuit of motor neurons and excitatory and inhibitory interneurons [52]. Contractions were scored at 0, 24 and 48 h after hatching over a 1-min period. Previous studies have identified that motor function defects become apparent after approximately 3 days in Smn mutant larvae [39,40]. At time point 0 and 24, both control and Smn mutants undergo a comparable number of contractions at 25˚C and 20˚C (Fig 1F and 1G). At larval age 48 h, the number of Smn mutant larvae contractions reduced by approximately 53% at 25˚C (Fig 1F) and 18% at 20˚C.
When compared to controls. Additionally, larvae were filmed for 1 min and tracked the distance travelled on an agar surface. Again, at 48 h, larvae travelled significantly less distance compared to controls (S1 Fig). At this time point, however, Smn mutant larvae are
significantly smaller than wildtypes and do not represent the same instar based on size or morphology. In summary, gross developmental defects, which include the pausing of neurogenesis, precede movement dysfunction in the larval SMA model.

**SMN knockdown in neurogenic cell types cause larval and adult locomotor dysfunction**

The coincidence of locomotor and developmental defects, and the ever-depleting SMN levels in *Drosophila Smn* mutants, make it difficult to identify the cellular mechanisms leading to the deterioration of neuronal function. With our interest in immature neuronal identities, the aim was to knockdown SMN by limiting Smn RNAi expression to neuroblasts and their daughter cells and to compare the results with pan-neuronal, neuronal subtype, glial cell and fat body Smn RNAi expression. Specifically, Smn knockdown was carried out using a RNAi hairpin construct SMN-RNAiN4. When expressed ubiquitously, SMN-RNAiN4 presents as a hypo-morph, with most flies dying at early pupal stage [39]. For expression in neuroblasts, the previously reported Inscurtable-GAL4 (Insc-GAL4) driver was used [53,54]. Insc-GAL4 is expressed in most embryonic and larval neuroblasts and their immature progeny [53], with a pattern of expression (S2A and S2B Fig) analogous to the enrichment of SMN observed in the post-embryonic neuroblasts [21] (S2C Fig). Expression of SMN-RNAiN4 removes the enrichment of SMN in these cells, although SMN is still present at low levels (S2D Fig). In addition, a driver containing a Prospero (Pros) regulatory sequence (P[pros-GAL4.U]) (Pros-GAL4) was acquired. Prospero protein drives the expression of neural differentiation genes and represses neuroblast stem cell identity and cell cycle proliferation genes. Prospero mRNA is expressed in the neuroblast, and the protein is asymmetrically localized to the neuroblast basal cortex during division, resulting in its partitioning into the daughter cells [55,56]. It was, therefore, speculated that the driver would express in neuroblasts and their daughter cells and, thus, provide an intermediary between Insc-Gal4 and drivers expressing pan-neuronally in differentiated neurons. To characterise the pattern of Prospero-GAL4 expression, UAS-CD8-GFP was driven using Pros-GAL4 and green fluorescent protein (GFP) localisation was noted. GFP was expressed in neuroblasts and daughter cells in the larval CNS (Fig 2A) and was restricted to only a few neurons in the adult thoracic ganglion (Fig 2B). To benchmark Insc-GAL4 and Pros-GAL4, two independent pan-neuronal drivers were used, namely elav-GAL4 and nSyb-GAL4. elav-GAL4 is expressed in all neurons, from newly born to mature, whereas nSyb-GAL4 expression is confined to mature neurons in which synapse formation has begun, or where synapses have been established. In addition, motor neuron (D42-GAL4 and OK371-GAL4), cholinergic neuron (Cha-GAL4), pan-glial (Repo-GAL4) and fat body (CG-GAL4) GAL4 drivers were used to manipulate SMN levels. The gross expression patterns of each driver are presented in Fig 2C. The number of larval body wall contractions was again scored when SMN was knocked down using the set of diverse neuronal drivers. As previously described, reduced body wall contraction defects were observed in pan-neuronal elav-GAL4; SMN-RNAi (Fig 2D), with an approximate 20% reduction in peristatic movements. No contraction defects were observed for nSyb-GAL4 or the motor neuron, cholinergic neuron, glial or fat-body drivers. Contraction defects were observed when SMN-RNAi was expressed in neurogenic cell types. A reduction in contractions was seen with both Insc-GAL4 and Pros-GAL4 expressed SMN-RNAi (15% and 14%, respectively (Fig 2D)). In addition, the effect of cell-type-specific Smn knockdown on survival to the adult stage was assessed. This was achieved by scoring the number of pupated larvae that emerged as adults (Fig 2E). As previously described, a reduction in eclosion rate was observed in pan-neuronally (elav-GAL4) driven SMN-RNAi (Fig 2E), with around 6% fewer flies eclosing in each case, compared with the two control groups.
Fig 2. Survival motor neuron (Smn) knockdown in neurogenic cell types leads to larval developmental defects and locomotor dysfunction. (A) Pros-GAL4 driven expression of membrane-bound CD8-green fluorescent protein (GFP) in the larval central nervous system. GFP expression is observed in the post-embryonic neuroblasts and their immature daughter cells; (B) Pros-GAL4 expression in the adult ventral nerve cord. No neurons within the thoracic ganglion show visible expression. Only a small number of neurons, which reside in the abdominal ganglion within the ventral nerve cord of the adult, expressed Pros-GAL4; (C) GAL4 nervous system expression patterns detailing the neuronal and glial cell type expression patterns; (D–E) SMN was knocked-down (UAS-SMN-RNAiN4) pan neuronally (Elav-GAL4 and nSyb-GAL4) predominantly in motor neurons (D42-GAL4 and OK371-GAL4), cholinergic neurons (Cha-GAL4), neuroblasts and undifferentiated daughter cells (Pros-GAL4 and Insc-GAL4), pan-glia (Repo-GAL4) and in the larval fat body (CG-GAL4); (D) body wall contractions were scored at 48 h, with significant differences observed with Elav-GAL4, Pros-GAL4 and Insc-GAL4 driven UAS-SMN-RNAi; ** and ***P < 0.01, **P < 0.00, Kruskal–Wallis test with Dunn’s multiple Comparisons; n > 20; (E) day of pupariation formation (three experiments each with n > 50; Kruskal–Wallis test with Dunn’s multiple comparisons) data showing that Insc-GAL4 and Pros-GAL4 SMN knockdown leads to a delay in time to pupariation; All error bars [SEM]. Scale bar = 10 μm.

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Furthermore, *Insc-GAL4* and *Pros-GAL4* expressed SMN-RNAi also decreased fly eclosion number, with around 7% and 8% less flies hatching, respectively.

Adult motor defects in the SMN knockdown lines were then assessed. Two phenotypes were selected to assess the effect on locomotion: adult activity and flight performance (Fig 3). *Drosophila* behaviour was analysed using adult flies over two 1-day periods using an environmentally controlled digital activity monitor (Chiu *et al.*, 2010) and by flight testing using the Seymour Benzer method [57]. For the full panel of drivers, activity and flight response was scored at 2 and 8 days after eclosion. As previously reported, flight and adult activity defects

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**Fig 3. Survival motor neuron (Smn) knockdown in neurogenic cell types leads to adult motor dysfunction.** Flies were tested for motor activity, (A) and (B), and flight ability (C) and (D) at 2 days (A) and (C), and 8 Days (B) and (D). *Drosophila* activity was detected in adult flies over 1 day using the Trikinetics activity monitors in controlled conditions; (A and B) only *Pros-GAL4/UAS-SMN-RNAiN4* and *Insc-GAL4/UAS-SMN-RNAiN4* progressively declined in activity over 2 (F; **P < 0.01, n = 20; Kruskal–Wallis test with Dunn’s multiple comparison) and 8 days (G; ***P < 0.001; n = 20; Kruskal–Wallis test with Dunn’s multiple comparison). (C and D) *Pros-GAL4; UAS-SMN-RNAiN4* and *Insc-GAL4; UAS-SMN-RNAiN4* flies showed a significant reduction in flight ability, with more flies residing at the bottom of the chamber, over 2 (F; *P < 0.05; ***P < 0.01, n = 40; Kruskal–Wallis test with Dunn’s multiple comparisons) and 8 days (G; ***P < 0.001; n = 40; Kruskal–Wallis test with Dunn’s multiple comparisons). All error bars [SEM].

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were observed when SMN-RNAi\textsuperscript{N4} was expressed using both pan neuronal drivers, nSyb-GAL4 and Elav-GAL4. No significant activity or flight defects, however, were observed when SMN-RNAi\textsuperscript{N4} was expressed in motor neurons, interneurons or sensory neurons at 2 or 8 days. In contrast, Pros-GAL4 and Insc-GAL4 driven SMN knockdown displayed a reduction in activity at 2 (Fig 3A) and 8 days (Fig 3B), and a progressive deterioration of flight ability at 2 (Fig 3C) and 8 days (Fig 3D). In summary, SMN reduction in neuronal progenitor cells, and with early to mature pan-neuronal expression, causes both larval and adult locomotor defects.

Motor defects persist with developmentally targeted spatiotemporal SMN knock down

Although GAL4 drivers generally express in defined cell types, transient or background expression may occur in other tissues or developmental stages. To limit this problem, and confirm the importance of the link between neuroblast and neuronal progenitor specific abnormalities and the motor dysfunction observed in the SMA model, SMN was knocked down with SMN-RNAi constructs using the GAL80\textsuperscript{TS} (TARGET) system [44]. The GAL80\textsuperscript{TS} system further refines transgene expression by targeting spatially confined GAL4 drivers to a specific developmental time period. In addition to the neuroblast-constrained driver (Insc-GAL4), the GAL80\textsuperscript{TS} system uses a temperature-sensitive GAL80 transgene (GAL80\textsuperscript{TS}) that represses GAL4 at low temperatures (e.g. 19˚C), but becomes inactive when the temperature is shifted to 29˚C, allowing GAL4 to be expressed [44]. This system was used to initially rear TubGAL80 and Insc-GALA/UAS-SMN-RNAi larvae at 29˚C (GAL80\textsuperscript{TS} is inactive; GAL4 and SMN RNAi is expressed). Larvae were then switched to 19˚C (GAL80\textsuperscript{TS} is active; the GAL4 gene is repressed; SMN RNAi is not expressed) at the start of pupation, to remove background SMN knockdown in differentiated adult neurons (Fig 3A). qRT-PCR control experiments, using a GFP reporter, highlighted that Insc-Gal4 did not drive GFP expression after switching to 19˚C (S3 Fig). Drosophila motor behaviour was again analysed using activity monitoring and flight testing, and an additional SMN RNAi construct was used (SMN-RNAi\textsuperscript{C25}). The use of SMN-RNAi\textsuperscript{C25}, which behaves as a hypermorph weaker than N4, enabled the comparison of two non-overlapping SMN RNAi constructs that have previously been shown to drive mild phenotypes [39]. Eight days after eclosing, both activity (Fig 4B) (SMN-RNAi\textsuperscript{N4}; SMN-RNAi\textsuperscript{C25}) and flight defects (Fig 4C) (SMN-RNAi\textsuperscript{N4}; SMN-RNAi\textsuperscript{C25}) were detected using this method. Therefore, it was confirmed that locomotor defects persist when SMN knockdown using neuroblast drivers is combined with the temporally restricted GAL80\textsuperscript{TS} TARGET system.

Smn expression in neuroblasts partially rescues survival and motor defects

Whether expression of SMN in the developing nervous system can rescue the larval locomotor function and survival defects observed in mutant Smn flies was next explored (Fig 5). The classic UAS-GAL4 system and the GAL80\textsuperscript{TS} (TARGET) system was used to drive full length SMN protein in the Smn mutants [58]. When using the GAL80\textsuperscript{TS} (TARGET) system, embryos were reared at 29˚C for the first 24 h, and then switched to 19˚C during larval life to restrict SMN expression to a period of embryonic neurogenesis (Fig 5A). qRT-PCR control experiments, using a GFP reporter, highlighted that Insc-Gal4 did not drive GFP expression after switching to 19˚C (S3 Fig).

Neuroblast expression of SMN (Insc-GALA/UAS-dSMN; Smn\textsuperscript{v7}/Smn\textsuperscript{A}) in Smn mutant larvae resulted in a marked improvement in embryo hatching defects, with 81% of expected observed compared with 59% observed in mutants alone (Insc-Gal4; Smn\textsuperscript{v7}/Smn\textsuperscript{A}). This result was consistent with the GAL80\textsuperscript{TS} (TARGET) system in which SMN replacement led to an improvement from 53% (TubGAL80\textsuperscript{TS}, Insc-GALA/UAS-dSMN; Smn\textsuperscript{v7}/Smn\textsuperscript{A}) of expected to 79% of expected (TubGAL80\textsuperscript{TS}, Insc-GALA/UAS-dSMN; Smn\textsuperscript{v7}/Smn\textsuperscript{A}) larval hatching.
In both cases, the compared control was formulated by normalising to the hatching number observed from an
Insc-GAL4/UAS-GFP cross. Tests were then conducted to see if neurodevelopmental restoration of SMN protein could also rescue larval locomotion and longevity defects. For locomotion, the peristaltic contractions in 72 h-old larvae, the time-point at which we see motor defects in Smn mutants, were counted. Control larvae (Insu-Gal4; UAS-GFP), which express GFP in neuroblasts in a wild-type background, underwent an average of 33 contractions per minute (Fig 5D). This contrasted with Smn mutant larvae, which underwent an average of 16 peristaltic contractions (Fig 5D) (Insc-GAL4; Smn<sup>x7</sup>/Smn<sup>A</sup>). When SMN was restored in Smn mutant neuroblasts (Insc-Gal4, UAS-SMN; Smn<sup>x7</sup>/Smn<sup>A</sup>), larval movement...
Fig 5. Restoration of survival motor neuron (SMN) in neurogenic cell types rescues the motor phenotypes in Smn mutant larvae. For rescue studies, both the classical binary GAL4 system (A) and the GAL80<sup>TS</sup> system (A') were used. For targeting, a temperature sensitive GAL80 (GAL80<sup>TS</sup>) represses GAL4 at 19˚C but becomes inactive at 29˚C. Embryos were reared for 24 h at 29˚C, during which GAL4 is expressed, then switched to 19˚C to eliminate expression. Expression of SMN using Insc-GAL4 rescue the embryonic attrition seen in SMN mutants with both the (B) binary and (C) TARGET GAL80<sup>TS</sup> GAL4 systems (*** <i>P</i> < 0.001, three experiments for each genotype, each with <i>n</i> = 60; Kruskal–Wallis test with Dunn’s multiple comparisons); (D) Insc-GAL4/UAS-SMN; Smn<sup>A</sup>/Smn<sup>x7</sup> larvae show significant rescue of locomotor activity at 72 h compared with mutant Insc-GAL4; Smn<sup>A</sup>/Smn<sup>x7</sup> (*** <i>P</i> < 0.001, <i>n</i> = 15, Kruskal–Wallis test with Dunn’s multiple comparisons); (E) larval survival was extended from a median of 3 days to a median of 7 days (three experiments for each genotype, each with <i>n</i> > 30; *** <i>P</i> < 0.001; Mantel-Cox); (F) Tub-GAL80<sup>TS</sup>; Insc-GAL4/UAS-SMN; Smn<sup>A</sup>/Smn<sup>x7</sup> larvae display a significant rescue of motor function at 72 h, compared with controls (*** <i>P</i> < 0.001, <i>n</i> = 20, Kruskal–Wallis test with Dunn’s multiple comparisons); (G) larval survival was extended from a median of 4 days to a median of 8 days (three experiments for each genotype, each with <i>n</i> > 30; *** <i>P</i> < 0.001; Mantel-Cox). All error bars [SEM].

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was significantly rescued, with 28 peristaltic contractions occurring on average (Fig 5D). An assessment of whether neuroblast expression of SMN in the Smn mutant background extended larval life was then made. The Smn mutant control larvae (Insc-GAL4; Smn<sup>x7</sup>/Smn<sup>A</sup>) lived for an average of 3.5 days (Fig 5E). When SMN was restored in Smn mutant larvae using the neuroblasts driver (Insc-GAL4, UAS-SMN; Smn<sup>x7</sup>/Smn<sup>A</sup>), average larval life was extended to 7.5 days. These larvae, however, did not pupate, and all died as larvae after 23 days.

To further validate these results, the GAL80<sup>TS</sup> system was again used to further refine the spatiotemporal window of UAS/GAL4 gene expression (Fig 5A). Smn mutant larvae with targeted Insc-GAL4 SMN expression (TubGAL80<sup>TS</sup>, Insc-GAL4/UAS-dSMN; Smn<sup>x7</sup>/Smn<sup>A</sup>) displayed significantly rescued peristaltic motor function (27 peristaltic contractions in 1 minute) compared with Smn mutants alone (TubGAL80<sup>TS</sup>; Insc-GAL4/++; Smn<sup>x7</sup>/Smn<sup>A</sup>), which had averaged 16 peristaltic contractions per minute. The larval longevity analysis was then repeated using the GAL80<sup>TS</sup> protocol (Fig 5A). When SMN was restored in Smn mutants using the neuroblasts driver (TubGAL80<sup>TS</sup>, Insc-GAL4/UAS-dSMN; Smn<sup>x7</sup>/Smn<sup>A</sup>), average larval life was extended to 7.5 days, when compared with Smn mutants alone, which only survived on average 4 days (TubGAL80<sup>TS</sup>; Insc-GAL4/++; Smn<sup>x7</sup>/Smn<sup>A</sup>). These results show that targeted addition of SMN protein to neuroblast cell populations can partially rescue motor function and longevity defects in the Drosophila SMA model.

### Combinatorial immature–mature neuron SMN knockdown synergistically enhances the locomotor and survival phenotypes

Irrespective of the effect of depleting SMN in neuroblasts and their immature daughter cells, loss of SMN in the mature motor system affects survivability and motor function [28,59,60]. The aim was to determine how depletion of SMN in the neuronal progenitor cell types and the mature nervous system enhances the motor and survival phenotypes. Dual SMN RNAi knockdowns were carried out using two combinations: 1) with Elav-GAL4, which expresses in both newly born and mature neurons, in conjunction with the neuroblast driver (Insc-GAL4); and 2) nSyb-GAL4, which expresses in maturing and mature neurons that have undergone synapse formation, in conjunction with the neuroblast driver (Insc-GAL4) (Fig 6A). To test these compound knockdowns, larval movement (Fig 6B) and the number of flies that survived to adulthood were analysed (Fig 6C). As previously described, Elav-GAL4 and Insc-GAL4 reduced peristaltic contractions, whereas nSyb-GAL4 SMN knockdown only led to adult movement phenotypes. Controls performed around 33 peristaltic contractions per minute, whereas SMN knockdown independently using Elav-GAL4, nSyb-GAL4 and Insc-GAL4 underwent approximately 26, 32 (non-significant) and 28 peristaltic contractions per minute, respectively (Fig 6B). When Elav-GAL4 and Insc-GAL4 were combined to drive SMN knockdown, larvae underwent 16 peristaltic contractions per minute on average, whereas compound nSyb-GAL4 plus Insc-GAL4 SMN knockdown larvae underwent 17 peristaltic contractions per minute on average (Fig 6B).

A similar trend was seen with eclosion (hatching from the pupal case; Fig 6C). Subtle but significant defects were observed in flies that underwent SMN knockdown with independent Elav-GAL4 and Insc-GAL4 expression, with flies hatching at 83% and 80%, respectively, and controls hatching at 90% (Elav-GAL4 + Insc-GAL4, 90%; and nSyb-GAL4 + Insc-GAL4, 90%) (Fig 6C). When Elav-GAL4 and Insc-GAL4, and independently nSyb-GAL4 and Insc-GAL4, were combined to drive SMN knockdown, hatching defects were enhanced (Elav-GAL4 + Insc-GAL4 driven UAS-SMN-RNAi<sup>N</sup>, 62% hatched; nSyb-GAL4 + Insc-GAL4 driven UAS-SMN-RNAi<sup>N</sup>, 60% hatched) (Fig 6C). These data demonstrate that the combinatorial knockdown of SMN in neuroblasts, immature neurons and mature neurons synergistically enhances the motor and survival phenotypes of the Drosophila SMA model.
In this study, we show that depleting SMN in neuroblasts and their immature daughter cells can predispose larval and adult *Drosophila* to locomotor dysfunction. In addition, we can partially rescue the larval motor defects of *Smn* mutants by restoring SMN in the neuroblasts and immature developing neurons using targeted expression systems. Finally, we highlight that the dual knockdown of *survival motor neuron* (SMN) in neuroblasts and differentiated neurons synergistically enhances the SMA model phenotypes.

![Diagram](https://doi.org/10.1371/journal.pgen.1010325.g006)

**Fig 6. Dual knockdown of survival motor neuron (SMN) in neuroblasts and differentiated neurons synergistically enhances the SMA model phenotypes.** (A) GAL4 nervous system expression patterns detailing the driver type and single and double-driver combinations used to knock down Smn. For negative controls Elav-GAL4 + Insc-GAL4 and *nsyb*-GAL4 + *Insc*-GAL4 were used. Elav-GAL4, Pros-GAL4 and *Insc*-GAL4 driven UAS-SMN-RNAiN4 were used as positive controls and compared with Elav-GAL4 + Insc-GAL4 and *nsyb*-GAL4 + *Insc*-GAL4 driven UAS-SMN-RNAiN4; (B) the genotypes were assessed for locomotor dysfunction at 72 h after hatching. Elav-GAL4 + Insc-GAL4 and *nsyb*-GAL4 + *Insc*-GAL4 driven UAS-SMN-RNAiN4 underwent reduced peristaltic contractions compared with negative and positive controls (**P < 0.001, n > 20, Kruskal–Wallis test with Dunn’s multiple comparisons**); (C) fly hatching number was then assessed to analyse survival to adulthood. Elav-GAL4 + *Insc*-GAL4 and *nsyb*-GAL4 + *Insc*-GAL4 driven UAS-SMN-RNAiN4 survived compared with negative and positive controls (**P < 0.001; three experiments, each with n > 50; Kruskal–Wallis test with Dunn’s multiple comparisons**). All error bars [SEM].

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**Discussion**

In this study, we show that depleting SMN in neuroblasts and their immature daughter cells can predispose larval and adult *Drosophila* to locomotor dysfunction. In addition, we can partially rescue the larval motor defects of *Smn* mutants by restoring SMN in the neuroblasts and immature developing neurons using targeted expression systems. Finally, we highlight that the
combination of presumptive and mature nervous system SMN reduction increases the severity of SMA model phenotypes. We show that the reduction of SMN in cells that are not synapse forming, and thus precede NMJ and sensory-motor network maturation, cause SMA-like phenotypes in the fly.

Although motor neuron loss is also a relatively late feature in SMA patients and mammalian models [25], it is believed that defects in synapse formation and maintenance may be central to the neurological phenotypes observed in SMA patients [59]. Mouse model rescue studies highlight that the therapeutic success of administered rescue constructs generally become progressively diminished only a few days after birth [31–36]. This pre- and peri-natal period coincides with a higher requirement of SMN level in the CNS, a phenomenon also observed in Drosophila [5,39,40]. It is difficult to compare the Drosophila life cycle with the vertebrate progression of disease; however, the mechanistic and cellular readouts from invertebrate models can offer some degree of translation. Our knockdowns and rescues are limited to neuronal stem cells and their immature progeny. Drosophila neuronal stem cells progress through a cascade of transcriptionally distinct identities before permanently differentiating or dying [45,46,61]. During division, this developmental cascade leads to a diversity of developmentally plastic immature daughter cells that undergo further pre- and post-transcriptionally regulated maturation steps, which precede the formation of synapses and ultimately action potentials. Although SMA was classically thought to be a disease of aberrant splicing, the broad requirement for SMN in the regulation of post-transcriptional gene expression is compelling, with roles encompassing snRNP biogenesis [1], mRNP biogenesis [62], mRNA transport [63], ribosomal dynamics [64], chromatin dynamics [65] and translational control [66]. It is probable that deficits in any one of these pathways could lead to stem cell or daughter cell sensitivity to conditions of low SMN. As a stem cell divides and creates a differentiating daughter cell, large changes in alternative splicing drive identity from one of pluripotency to that of an identifiable neuronal lineage with a defined cell biology and physiology [67,68]. It may be that higher SMN levels are required for the temporal–spatial regulation of the alternative splicing events that occur during this switch. Provisional data has shown that both adult flies and larvae display fewer synapses and synaptic boutons respectively, when SMN is knocked down in neuroblasts and the corresponding immature progeny. However, the relevance of bouton number changes has been partly called into question, and these alterations may only be casually linked to the movement defects physiological alterations, and death, observed in Drosophila SMA models [49,69]. It may be that upstream functional changes in motor neurons, interneurons, or other neuronal cell types may ultimately lead to the degeneration of the motor neuron or the neuromuscular junction. To this end, the temporal transcription factor cascades that generate the molecular and physiological diversity of the neurons in the developing CNS may be of interest [46,61]. In future work, we would like to see, when SMN levels are low, if molecular changes in the developing neurons lead to defects in motor neuron physiology, or alterations in the different neuronal classes. We can speculate that changes at this level could alter, in a subtle manner, their molecular identity sensitising neurons to degeneration in certain conditions, or over time.

We have previously reported that SMN overexpression affects developmental timing in Drosophila [21] and protects embryonic stem cells from retinol-induced differentiation [19]. In in-vivo SMN mutant neuroblast clones, the levels of both major and minor spliceosome snRNPs (U5 and U2) are reduced in the nucleus of the neuroblast. We have also shown that SMN loss in neuroblasts perturbs cell division and alters the topology of the daughter cell cluster. Furthermore, gene expression analysis conducted on the spinal cord from SMN deficient mice detected changes in proliferative pathways, and identified morphological changes in the dividing cells in the ventral horn [70]. In both mouse and Drosophila models, SMN reduction...
promotes the untimely differentiation of neurons and spermatogonia \cite{19-21}, suggesting that high SMN is required for the fidelity of the developmental processes key to cellular differentiation and maturation.

Alternatively, or in-combination, SMN loss may affect downstream translational control. We have previously shown that SMN-deficient neuroblasts display a mislocalisation of a cortical scaffolding protein that binds asymmetrically localised RNP complexes. *Drosophila* neuronal stem cells and neurons alike are energetically demanding, polarised and metabolically distinct \cite{71}; therefore, the presence of highly clustered sites for RNP maturation and processing may be necessary for the correct function of metabolically active and dividing cell types \cite{17}. Nevertheless, it is important to note that cell types and tissue beyond the nervous system are affected by SMN loss \cite{72}. Within our model, although we rescue motor phenotypes and expand lifespan, neuroblast SMN rescue cannot support full larval development, pupariation and pupation to adulthood. In addition, SMN depletion in mature neurons also leads to locomotor dysfunction. Mutations in many widely expressed genes cause selective neuropathies and motor neuron diseases \cite{73}. How we come to think about the nature of these disease genes, particularly their pleiotropy and spatial and temporal requirements, will be key to the eventual revealing of the mechanisms that lead to the selectivity of cell type degeneration.

It seems that, at least in the *Drosophila* model, SMN reduction in many presumptive and mature neuronal cell types can cause locomotor defects and reduce lifespan. To understand the temporal and spatial requirement of SMN during nervous system development, we used *Drosophila* cell and time-specific GAL4 drivers. To date, a large number of UAS/GAL4 studies have investigated how to identify the fly tissue and cell types sensitive of SMN loss \cite{5}. Consistent with other models, ubiquitous SMN knockdown is the most severe, leading to larval lethality, whereas ubiquitous rescue using high level expression drivers confers adult survival \cite{39,40}. Second to this, combinatorial experiments expressing SMN, both early stage pan-neuronal and mesoderm drivers, partially rescues at the adult stage, whereas knockdown using the same driver combination causes larval–pupal lethality \cite{40}. In comparison, pan-neuronal knockdown alone leads to modest adult lethality, neurophysiological and behavioural defects \cite{39,40,74}, whereas a subset of other drivers, including those expressed in cholinergic neurons and glutamatergic neurons, have shown specific neuromuscular phenotypes or rescue profiles \cite{41,74}. It is important to highlight that GAL4 drivers will vary in temporal specificity and level. Due to the non-synchronous correlation between transcriptome and proteome \cite{75}, enhancer drivers derived from known neuronally expressed genes may generally express earlier or more broadly (at least at lower levels) than expected. These issues highlight the importance of the GAL80\textsuperscript{TS} system used in this study to confine transgene expression to the cell type and time period. However, it is important to note that although the target system offers a high degree of temporal and special control, our study does not fully eliminate the role of low level SMN knockdown and expression in other tissues enhancing the phenotypes and rescues observed, during the period of GAL4 expression.

To summarise, the present research supports that the idea that SMA is caused by a combination of defects that impact motor neuron development, maturation, and maintenance. Moreover, although motor neurons seem to be particularly sensitive to SMN loss, the complex background of multiple tissue defects makes it difficult to unveil the precise timing and nature of the causative defects. The present study directly shows that SMN is required during a window of neurogenesis that precedes synapse formation and neuromuscular junction maturation, and that the motor defects observed in *Drosophila* SMA models can be, in part, be caused by SMN reduction in these cell types. To this end, further study should address how an improper set-up of neuronal networks may compound any motor neuron cell autonomous defects that may arise in SMN-deficient motor neurons.
**Materials and methods**

**Drosophila husbandry and stocks**

*Smn*\(^{x7}\) null, *Smn\(^{A}\)*, P[UAS-Smn-RNAi]N4, P[UAS-Smn-RNAi]\(^{C25}\) line have been previously described [39, 40]. All stock were backcrossed onto *w\(^{118}\)* wild type background. Larvae were grown on apple juice plates with yeast and rich food added. Low population density was maintained for all crosses prior to experimentation. For classical UAS/GAL4 experiments, all crosses were carried out at 25˚C to generate extensive but not complete knock-down. GAL4 drivers 1032-GAL, D42-GAL4, OK371-GAL4, Cha-GAL4, Repo-GAL4, and CG-GAL4 drivers, were obtained from Bloomington (Indiana). Pros-GAL4 was obtained from the putative-enhancer collection (Bloomington Drosophila Stock Centre [BDSC] at Indiana University, USA). Insu-GAL4; Tub84B-GAL4\(^{TS}\) was a gift from Jürgen Knoblich. Drivers were characterised using UAS-CD8-GFP, UAS-H2B-YFP (Andrea Brand).

**EdU staining**

Dissected CNS were incubated for 1.5 h in 10 μM EdU/Grace’s medium, fixed for 10 min in 4% paraformaldehyde, followed by detection of Alexa Fluor azide according to the Click-iT EdU Imaging Kit (Invitrogen, Waltham, MA, USA) and washing in 0.2% Triton X-100 in phosphate buffered saline. Immunofluorescence was carried out as previously described [21].

**Larval hatching assays**

A 2-h lay was carried out on apple juice plates and embryos were lined up in sets of 10. The number of embryos that hatched into larvae was scored for each genotype and was expressed as a percentage of that expected from the lay. *Smn*\(^{x7}\)/TM6B-GFP and *Smn*\(^{A}\)/TM6B-GFP were crossed and the number of embryos and larvae with *Smn* heterozygotes) and without GFP expression (homozygous *smn* mutants) were scored as a percentage.

**Larval locomotion assays**

Measurement of motor function involved placing individual age-matched third instar larvae at the centre of a 0.7% (weight by volume) agar plate and counting the forward body wall contractions exhibited over 1 min. Larvae were left to acclimatise for 30 s before analysis.

**Larval survival assay**

Flies performed a 2hr lay on apple juice plates for 2-h with minimal yeast. Embryos were counted, additional yeast was added, and larval development and death was recorded every 24 h.

**Adult locomotor function assay**

Age-matched (1- and 7-day old) male flies were placed individually in a 5-mm glass activity tube containing a food source (5% sucrose [Sigma-Aldrich, St Luis, MO, USA] and 2% Bacto agar [BD Diagnostics, Franklin Lakes, NJ, USA] in distilled water) at one side and a plastic cover with an air hole at the other. The individual glass tubes were placed into the activity monitor (Trikinetics monitors DAM2) (Trikinetics Inc., Waltham, MA, USA) and supported with rubber bands to hold them in place. Locomotor activity was recorded when the flies crossed the infrared light beam at the middle of the glass tubes. Thirty flies were used per genotype and kept under controlled conditions (12-h light–dark cycle at 25˚C) for 2 days, day 1 being excluded for habituation. The DAM System collection software was used for collecting
data. The raw binary data were processed using DAM Filescan102X (Trikinetics Inc., Waltham, MA, USA) and summed into 1-h bins.

**Adult flight assay**

The flight assay was carried out in accordance with a modified protocol originally designed by Benzer [57]. A total of 1000-ml graduated cylinder divided into five sectors was coated internally with mineral oil. Flies were introduced into the top of the cylinder through a funnel and the flies stuck in each sector were counted. The height that flies stick in the cylinder is indicative of their flight capabilities.

**TubGAL80<sup>TS</sup> TARGET analysis**

For larval rescue analysis, GAL80<sup>TS</sup> analysis, embryos were reared at 29˚C (GAL80<sup>TS</sup> inactive; GAL4 active) and after 24 h (GAL80<sup>TS</sup> active; GAL4 repressed) and then switched to 19˚C during larval life. TubGAL80<sup>TS</sup>, Insc-GAL4/UAS-dSMN; Smn<sup>x7</sup>/Smn<sup>A</sup> stock was used and compared with the mutant TubGAL80<sup>TS</sup>, Insc-GAL4/+; Smn<sup>x7</sup>/Smn<sup>A</sup> and control TubGAL80<sup>TS</sup>, Insc-GAL4/UAS-GFP backgrounds. For adult analysis, *Drosophila* larvae were reared at 29˚C (GAL80<sup>TS</sup> inactive; GAL4 active) and then switched to 19˚C (GAL80<sup>TS</sup> active; GAL4 repressed) after pupariation formation. Two non-overlapping RNAi construct was used (SMN-RNAi<sup>N4</sup> and SMN-RNAi<sup>C25</sup>) and expressed using a TubGAL80<sup>TS</sup>, Insc-GAL4 stock line. *Drosophila* motor behaviour was analysed using activity monitoring, which was carried out at 19˚C at days 1 and 7 after hatching. Flight testing was carried out at 8 days.

**qRT-PCR**

We determined the levels of GFP mRNA using qPCR methods as described previously [76], using Fast SYBR Green Master Mix (Applied Biosystems Cat. no. 4385612) and the 500 Fast Real-Time PCR System (Applied Biosystems).

**Statistical analysis**

A Kruskal–Wallis test and subsequent Dunn’s multiple comparison testing were carried out unless otherwise stated. GraphPad Prism software was used for all data analysis.

**Supporting information**

**S1 Fig. Movement defects present at late larval stages.** (A) Control (w<sup>1118</sup>) and Smn<sup>x7</sup>/Smn<sup>A</sup> larvae were monitored at approximately 24, 48, and 72 ± 1 h after egg laying. Acclimatised larvae were filmed for 1 min, and the distance travelled was traced and measured in cm. Smn<sup>x7</sup>/Smn<sup>A</sup> larvae displayed significant movement defects at 72 h (***P < 0.001, n = 20); (B) example superimposed larval locomotion path traces from control and Smn<sup>x7</sup>/Smn<sup>A</sup> mutants for each time point.

(TIF)

**S2 Fig. Characterisation of Insc-GAL4 expression and targeted SMN knockdown.** (A) During larval life, a second wave of larval neuroblast division occurs. The majority of neuroblasts in the ventral ganglion reside at the surface of the larval CNS. (B) Representative Inscu-GAL4 expression is seen exclusively in neuroblasts and immature neurones in the ventral ganglion and brain lobes. Insc-Gal4 expression was examined using UAS-mCherry. The ventral and medial regions of a third instar larval central nervous system is shown. (C) The larval CNS were co-stained with SMN. The zoom (Box in B) shows SMN staining overlaps with UAS-mCherry immunofluorescence. (D) Expressing UAS-SMN-RNAi<sup>N4</sup> exclusively in neuroblasts
and immature neurones significantly reduces, but does not eliminate, SMN levels. Edu staining highlights a population of dividing neuroblasts and ganglion mother cells that no longer show SMN enrichment.

S3 Fig. Relative expression of GFP mRNA normalised to rp49 in Tub-GAL80<sup>TS</sup>; Insc-GAL4/UAS-GFP during the larval and adults experimental time courses. (A) The GAL80<sup>TS</sup> system was used to eliminate any adult GAL4 expression. For larval experiments, a temperature sensitive GAL80 (GAL80<sup>TS</sup>) represses GAL4 at 19˚C but becomes inactive at 29˚C was used. Embryos were reared for 24 h at 29˚C, during which GAL4 is expressed, then switched to 19˚C to eliminate expression. (B) GFP RNA was measured in whole embryos and larval CNS over the time course analogous to that used in the locomotor and pupation assays. GFP expression was seen to diminish by 0 hrs. We detected no further GFP expression throughout the course of the experimental period. (C) For adult studies, larvae were reared at 29˚C (GAL80<sup>TS</sup> is inactive; GAL4 is active) and then switched to 19˚C (GAL80<sup>TS</sup> is active; GAL4 is repressed) at the start of pupation. (D) Relative expression of GFP mRNA normalised to rp49 in Tub-GAL80<sup>TS</sup>; Insc-GAL4/UAS-GFP larvae, pupae and adults. GFP RNA was measured in larval, pupal and adults over the time course analogous to that used in the adult activity and flight assays. The Larvae were switched from 29 to 19˚C at the late L3 stage. GFP expression was seen to diminished during larval growth and maturation. We detected no GFP expression throughout the pupal and adult periods studied. (L2, 2<sup>nd</sup> Instar Larvae; L3, 3rd Instar Larvae).

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