Mushroom body defect is required in parallel to Netrin for midline axon guidance in Drosophila

Sophie Cate¹, Sangeetha Gajendra¹, Samantha Alsbury¹#, Thomas Raabe², Guy Tear¹§ and Kevin J Mitchell³,⁴*.

(1) MRC Centre for Developmental Neurobiology, New Hunts House, King’s College, London SE1 1UL, UK.
(2) MSZ Universitat Würzburg, Versbacher Strasse 5, 97078 Würzburg, Germany
(3) Howard Hughes Medical Institute, Division of Neurobiology, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA
(4) Smurfit Institute of Genetics and Institute of Neuroscience, Trinity College Dublin, Dublin 2, Ireland.

* Joint senior authors

# current address Department of Life and Sports Science, University of Greenwich, Medway Campus, Chatham Maritime, Kent ME4 4TB, UK.

§ corresponding author

MRC Centre for Developmental Neurobiology,
New Hunts House, King’s College, London SE1 1UL, UK
guy.tear@kcl.ac.uk
Tel: +44 207 848 6539
Fax: +44 207 848 6550

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Summary

The outgrowth of many neurons within the central nervous system is initially directed towards or away from the cells lying at the midline. Recent genetic evidence suggests that a simple model of differential sensitivity to the conserved Netrin attractants and Slit repellents is not sufficient to explain the guidance of all axons at the midline. In the *Drosophila* embryonic ventral nerve cord, many axons still cross the midline in the absence of the *Netrin* genes or their receptor *frazzled*. Here we show that mutation of *mushroom body defect* (*mud*) dramatically enhances the phenotype of *Netrin* or *frazzled* mutants, resulting in many more axons failing to cross the midline, though mutations in *mud* alone have little effect. This suggests that *mud*, which encodes a microtubule-binding coiled-coil protein homologous to NuMA and Lin-5, is an essential component of a Netrin-independent pathway that acts in parallel to promote midline crossing. We demonstrate that this novel role in axon guidance is independent of Mud’s previously described role in neural precursor development. These studies identify a parallel pathway controlling midline guidance in *Drosophila* and highlight a novel role for Mud potentially acting downstream of Frizzled to aid axon guidance.
Introduction

In the central nervous system (CNS) of vertebrates and invertebrates most neurons extend across the midline to form commissures while the remainder extend on their own side (Tear, 1999; Kaprielian et al., 2001; Garbe and Bashaw, 2004; Evans and Bashaw, 2010). This decision depends in part on the responsiveness of the growth cone to Netrin attractants and Slit repellents, both secreted from cells at the midline, though additional mechanisms also exist to direct axons across the midline (Andrews et al., 2008; Dickson and Zou, 2010; Evans and Bashaw, 2010; Spitzweck et al., 2010; Organisti et al., 2015).

The Netrins act as chemoattractants to bring axons to the midline in flies, worms and vertebrates. In all these organisms, netrin loss-of-function causes defects in the projection of axons towards the midline (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996) and the same is true of mutations in the Netrin receptors, unc-40, DCC and frazzled (Hedgecock et al., 1990; Kolodziej et al., 1996; Fazeli et al., 1997). However their activity does not fully account for the guidance of all commissural axons across the midline suggest existence of additional mechanisms (Brankatschk and Dickson, 2006).

In Drosophila, a number of components of Netrin-independent mechanisms that attract axons to the midline have been identified. Removal of either Dscam, fmi or robo2 significantly enhances the failure of midline crossing caused by the absence of frazzled alone (Andrews et al., 2008; Spitzweck et al., 2010; Organisti et al., 2015). However, the loss of any of these genes individually does not lead to a significant midline guidance defect. Thus the role of these additional pathways in directing commissural axons across the midline is only revealed in the absence of the Netrin signalling.
Here we show that Mushroom Body Defect (Mud) also has a role in a Netrin-independent signalling pathway directing commissural axons to the midline in Drosophila. Mud has previously been identified to function within neuroblasts and sensory organ precursors to couple the orientation of the mitotic spindle to both intrinsic and extrinsic cues (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Siller and Doe, 2009; Segalen et al., 2010). We show that its role in axon outgrowth is independent of its activity within neuroblasts. Mud is expressed within post-mitotic neurons where it may act downstream of Frizzled to influence intrinsic neuronal polarity necessary for axonal outgrowth.
Materials and methods

Genetics

The following Drosophila stocks were used: (1) mud¹/Fm7cβGal, (2) mud³/Fm7cβGal, (3) fra⁹⁵⁷/CyOwgβGal, (4) Df-NPS/Fm7cβGal, (5) Df-KA9/Fm7cβGal, (6) NetA,B/Fm7βactin (courtesy of B. Dickson, Vienna), (7) fz¹/Tm6bAbdA lacZ, (8) mud¹/Fm7c; fra⁹⁵⁷/CyOwgβGal, (9) Pins⁶²/Tm6bAbdA lacZ, (10) fra⁹⁵⁷/ CyOwgβGal; PinsP62/Tm6bAbdA lacZ, (11) mud²/Fm7cβGal; Pins⁶²/Tm6bAbdA lacZ, (12) P[EPgy2]EY20197, (13) egGal4::UASCD8GFP, (14) elavGal4 on II, (15) egGal4, (16) Sca-GAL4, (17) mud³/Fm7kr::GFP, (18) fra⁹⁵⁷/CyODfΔEYFP. X;Y translocation stocks with breakpoints in the 12E-13A region, originally constructed by Stewart and Merriam (1973) were used to generate deficiencies for defined regions of the X chromosome as described by (Ashburner, 1989). Unless otherwise stated, stocks were obtained from the Bloomington Stock Center.

Molecular Biology

A genomic rescue construct for mud was created in P[acman] (Venken et al., 2006). 17.5kb was retrieved from BAC CH322- 147E14 (BACPAC Resources Center) (Venken et al., 2009) covering chromosome arm X from 14138384 to 14157868. The rescue construct includes the promoter region of mud, located 147bp downstream of CG32599 to 1546bp upstream of mud, the mud gene and region downstream to 363bp upstream of the closest downstream gene, CG1461. 500bp homology arms homologous to the left and right ends of the transgene were subcloned into P[acman] to create a targeting construct. MW005 cells (courtesy of Colin Dolphin, King’s College, London, described in (Westenberg et al., 2010)) were made competent for recombineering by inducing the Red recombinase essentially as described in (Dolphin and Hope, 2006). After verification of the correct integration of genomic DNA into P[acman] by sequencing, transgenic flies containing this P[acman]-mud construct inserted into the VK6 attP site at 19E7 on the X chromosome were obtained by BestGene Inc. USA. A venus-YFP tag was inserted at the N terminus of mud using Gibson assembly and the construct inserted at attP40 by Bestgene.

Immunohistochemistry

Embryos were collected, fixed and stained as previously described by Kidd et al, (1998). The following primary antibodies were used, Mab BP102 (Developmental Studies Hybridoma Bank (DSHB); 1:20), Mouse-anti-βGal (Promega; 1:300), Mouse-anti-connectin courtesy of Robert White (University of Cambridge, UK) (Meadows et al., 1994); 1:20 and Rabbit-anti-GFP (Invitrogen; 1:300). Secondary
antibodies were purchased from Molecular Probes. Stacks of images were obtained using a Zeiss LSM 510 confocal processed using Volocity 5.2 imaging software (Improvision, UK).
Results and Discussion

*Netrin* deficiencies reveal the presence of an additional activity mediating axon guidance across the midline.

*Drosophila* has two *Netrin* genes, *NetA* and *NetB*, which are adjacent on the X chromosome (Fig. 1A). The original studies investigating the role of the *Drosophila Netrins* reported a difference in phenotype between a small deficiency Df(1)NP5 that removed both *Netrin* genes and a slightly larger deficiency Df(1)KA9 that extends further than NP5 (Fig 1A) (Harris et al., 1996; Mitchell et al., 1996). Embryos hemizygous for the smaller deficiency Df(1)NP5 have thinner or occasionally absent axon commissures in the ventral nerve cord, with the posterior commissure being more strongly affected, and occasional breaks in the longitudinal connectives (Fig. 1B, Table 1). This phenotype is similar to that seen in embryos where only *NetA* and *NetB* have been removed (Fig 1B, Table 1) (Brankatschk and Dickson, 2006), although the Df(1)NP5 is slightly more severe (Andrews et al., 2008). In contrast, embryos hemizygous for the slightly larger deficiency Df(1)KA9 exhibit a more severe phenotype with a near complete loss of midline crossing in some commissures (Fig 1B, Table 1). The larger deficiency affects the guidance of anterior and posterior commissural axons at the midline.

Restoration of either *Netrin* gene at the midline is sufficient to completely rescue the Df(1)NP5 phenotype while rescuing the Df(1)KA9 to near wild type (Harris et al., 1996; Mitchell et al., 1996). These findings indicate the existence of a gene activity also deleted in Df(1)KA9 that enhances midline crossing defects caused by the absence of the *Netrins*, but which has a mild phenotype when removed alone.
Markers for specific subsets of commissural neurons confirm the increased severity of commissural defects in Df(1)KA9 compared to Df(1)NP5 embryos (Table 1) (Fig 2). The Eg-GAL4 driver identifies the EG cluster of 10-12 cells that extend axons in the anterior commissure and the EW cluster of four cells that project in the posterior commissure (Higashijima et al., 1996; Dittrich et al., 1997; Garbe and Bashaw, 2007). Midline crossing by eagle-positive EG and EW neurons is significantly more disrupted in Df(1)KA9 than in Df(1)NP5, due both to stalling of axons prior to midline crossing and to misguidance where axons extend across the midline along an aberrant trajectory (Table 1) (Fig 2). In Df(1)NP5 embryos the EW axons fail to cross the midline in 37% of segments while 20% of the EG axons do not cross. In Df(1)KA9 embryos the number of segments where EW axons fail to cross the midline is increased to 78% (Fig 2). The outgrowth of the SP1 neuron, one of the earliest axons to cross the midline in the anterior commissure was examined using anti-Connectin (Meadows et al., 1994). Behaviour of the SP1 neurons mirrors that of the EG axons, with significantly more failing to cross the midline in Df(1)KA9 (57%), compared to Df(1)NP5 (21%).

**Mud is the enhancer of Netrins**

We used overlapping synthetic deficiencies in the region (Livingstone, 1985) to map the enhancer activity (Fig. 1A). Df(1)B24-B128, which deletes both NetA and NetB plus distal material, displays the stronger axon guidance phenotype suggesting the gene responsible lies distal to NetA. The synthetic deficiency Df(1)B24-B54 selectively removes this distal genetic material – which includes a candidate gene, mushroom body defect (mud) and a small number of additional genes – while leaving the Netrin genes intact. Embryos hemizygous for this deficiency display a subtle CNS axon pathway phenotype. There is a general but weak
irregularity in the usually orthogonal organisation of axon tracts as revealed by BP102, with occasionally thinner commissures and rare breaks in the longitudinals. This phenotype is indistinguishable from that observed in embryos hemizygous for any of several alleles of mud (Fig. 1B).

To test whether mud encodes the additional midline guidance activity removed in Df(1)KA9 we examined embryos double mutant for mud and frazzled. Frazzled embryos have a similar commissural axon guidance phenotype to small Netrin deficiencies (Fig. 1B) (Kolodziej et al., 1996). When mud alleles, or the small deficiency Df(1)B24-B54 that deletes the mud region, are combined with frazzled alleles the double mutant embryos fail to form the majority of commissures - a phenotype indistinguishable from that of Df(1)KA9.

Confirmation that mud is necessary for the formation of the commissures that form in Df(1)NP5 embryos was demonstrated by reintroducing mud as a transgene into Df(1)KA9 embryos, using a BAC construct that contains the mud genomic region. This resulted in a rescue of the BP102 phenotype in Df(1)KA9 embryos to one that resembles that in the smaller Df(1)NP5 deficiency (Fig. 1B). Thus mud encodes the enhancer activity that accounts for the more severe phenotype observed in the larger Df(1)KA9 deficiency and is necessary to enable axons to cross the midline in this background. This places Mud as a component within an additional signalling pathway that directs axons to the midline, the role of which becomes apparent in the absence of Netrin signalling.
Mud mutation has direct effects on axon extension and guidance

Mud has previously been shown to be required during the asymmetric division of embryonic neuroblasts, where it couples mitotic spindle orientation to cortical polarity at metaphase (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Initial defects in this process in mud mutants are largely recovered by a re-alignment of the spindle during telophase, and only minor consequences have been reported on subsequent neuronal number and fate. The pattern of expression of even-skipped is largely unchanged in mud mutants, Df(1)KA9 or Df(1)NP5 (Izumi et al., 2006) (data not shown). Similarly, the neurons identified by anti-Futsch (22C10) and anti-Fasciclin-II (1D4) form as normal (data not shown), indicating little or no change in cell fate. To test further whether Mud’s role during neuroblast division and axon outgrowth are separable, the ventral nerve cord phenotypes of fra;pins double mutants were examined. Pins functions with Mud in asymmetric neuroblast division (Siller et al., 2006) and should loss of mud during neuroblast division lead to defects in subsequent axon outgrowth, a pins mutant should have a similar effect on axon guidance and would enhance fra phenotypes. However, the ventral nerve cord phenotype of fra;pins double mutants is no more severe than that of fra mutants alone (Fig 3A). The effects on axon guidance due to absence of Mud are thus not attributable to a disruption of cell fate.

We also examined the consequence of manipulating the levels of Mud activity within neuroblasts and neurons using the UAS-GAL4 system (Brand and Perrimon, 1993). To do this we made use of a P-element insertion (EY20197) that inserts a UAS immediately upstream of mud. Increasing Mud expression in neuroblasts using the Sca-GAL4 driver did not result in axon outgrowth defects (data not shown). However, increasing Mud expression in the Eagle-neurons caused a reduction in the number of axons extending across the midline through the
anterior commissure (Fig 3B) suggesting that Mud acts in a dose dependent manner in axons. The decrease in axons is not due to a loss of the cells. This disruption is consistent with a direct role of Mud in axonal projection or guidance in post-mitotic neurons.

**Mud is expressed in post-mitotic neurons**

*Mud* encodes a protein that contains multiple coiled-coil domains and a microtubule-binding domain and which shares similarity to the vertebrate protein NuMA and lin-5 of *C. elegans* (Guan et al., 2000; Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). The *Drosophila* gene encodes seven isoforms and a probe which detects all isoforms shows that mud transcripts are expressed throughout embryonic development. Zygotic expression is restricted to the ventral nerve cord from stage 11 and mud remains expressed in the ventral nerve cord until the end of embryogenesis.

Mud protein expression has previously been described to be localised to both the apical cortex and the centrosome of neuroblasts (Izumi et al., 2006). We find that Mud is also expressed within neurons where Mud expression is localised within a punctate pattern within the soma. Mud is expressed in most, if not all, neurons and is also present within midline cells and members of the longitudinal glia (Fig 3C). NuMa has similarly been reported to be expressed in a particulate distribution within the somatodendritic compartment of postmitotic sympathetic and hippocampal neurons, a distribution that requires intact microtubules (Ferhat et al., 1998). Mud, in common with its homologues, lin-5 in *C. elegans* and vertebrate NuMA, is able to bind microtubules, has a conserved role in regulating mitotic spindle formation and is able to link intrinsic or extrinsic cues to orient spindle formation (Du et al., 2001; Segalen et al., 2010). Mud also has an ability to recruit dynein/dynactin (Siller
and Doe, 2009) and functions in the planar cell polarity pathway (Segalen et al., 2010; Johnston et al., 2013), raising the possibility that Mud may function in neurons to link polarity information with the dynein/dynactin complex to orient microtubule structures within neurons to encourage directed outgrowth.

**Commissure formation utilises a variety of partially redundant pathways**

Multiple signalling pathways in addition to Netrins cooperate to direct axon outgrowth towards and across the midline. Mutations in the genes encoding the transmembrane proteins Dscam, Robo2, or Flamingo or the intracellular proteins Abelson and Trio and now Mud can all dramatically enhance the reduction of axonal midline crossing in Netrin or frazzled mutants (Forsthoefel et al., 2005; Andrews et al., 2008; Spitzweck et al., 2010; Evans et al., 2015; Organisti et al., 2015). Because Mud has previously been implicated in a signalling pathway downstream of Frizzled, in planar cell polarity (Segalen et al., 2010), we tested for genetic interactions between frizzled and Netrin and between frizzled and mud. We find, as recently independently reported (Organisti et al., 2015), that NetAB;fz double mutants display a severe lack of commissures, similar to mud;fra or Df(1)KA9 mutants, whereas mud;fz mutants are not appreciably more severe than either mud or fz alone (Fig 3D). These data are consistent with a model where Fz and Mud both operate in a common, parallel pathway to the Netrins, possibly in concert with Fmi (Organisti et al., 2015). Double mutants of mud with Robo2 or with Dscam did not show a significant increase in midline crossing defects (data not shown), which formally suggests that Mud may also act in common pathways with these proteins, but this straightforward interpretation is complicated by the multiple functions demonstrated for Robo2 and Dscam in midline guidance (Andrews et al., 2008; Evans et al.,
Further investigations are necessary to gain a better understanding of the mechanisms through which these multiple pathways are integrated within growth cones to enable the precise navigation of commissural axons at the midline.
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Figure 1

Identification of mud as an additional axon guidance factor required for commissure formation in the Drosophila CNS.

(A) Schematic representation of the regions of the X chromosome deleted by the deficiencies Df(1)KA9 and Df(1)NP5 (open boxes) used to remove the two Netrin genes, NetA and NetB. Distal is to the left. Bracketed lines below represent the extents of the synthetic deficiencies used in this study that identify the location of an additional activity required for midline crossing distal to the Netrin genes. (B) Drosophila embryos immunostained with the CNS axon marker Mab BP102, anterior up. In the wild type embryo axon pathways extend in an orthogonal pattern with longitudinal tracts positioned either side of the midline and a pair of commissural tracts that connect the two sides of the nervous system within each segment. In embryos bearing double mutations for NetA and NetB commissure formation is disrupted with fewer axons attracted across the midline, with the posterior commissure affected more severely. Embryos homozygous for a chromosomal deficiency, Df(1)NP5, that removes the Netrin genes have a phenotype similar to that of NetA, NetB animals, while a slightly larger deficiency Df(1)KA9 has a stronger BP102 phenotype with fewer axons attracted to the
midline suggesting an additional activity has been removed. The synthetic deficiency Df(1)B24-B128 that is deficient for the Netrin genes and a distal region display the stronger phenotype. Embryos deficient for the distal region alone, Df(1)B24-B54 display very little disruption to the axon pathways. Mutations that remove the attractive Netrin receptor frazzled have a similar phenotype to that in embryos lacking the Netrin genes. When loss of frazzled is combined with a removal of the distal material Df(1)B24-54; fra cause the increased midline crossing failure phenotype. Mud is a candidate gene for the addition activity removed in Df(1)B24-54, mud;fra double mutations display the same enhanced phenotype as in Df(1)KA9 embryos. Reintroduction of mud as a transgene into Df(1)KA9 embryos reverts the midline phenotype to that seen when the Netrin genes are removed alone and also rescues the minor phenotype seen in mud mutant animals confirming that mud encodes the additional midline attractive activity.
Loss of mud enhances axon outgrowth defects at the CNS midline in netrin deficient embryos.

(A) Eagle-positive axons extend in the anterior (EG) and posterior (EW) commissures at the midline of the CNS. (B) Upon loss of Netrin signalling (Df(1)NP5) there is a reduction in ability of eagle-positive axons to cross the midline leading to a thinning of commissures (arrowhead) or complete loss of midline crossing (arrow). (C) Loss of both Mud and Netrin activity (Df(1)KA9) results in a greater disruption of midline crossing with many axons failing to cross in both anterior and posterior commissures (arrow) or taking aberrant trajectories.
(arrowhead). (D) Anti-Connectin reveals SP1 axons that extend in the anterior commissure (open arrow) and additional axons extending in the posterior commissure (arrowhead). (E) Loss of mud alone leads to mild defects with 7% of segments showing failure of SP1 axons to cross. (F) Loss of Netrin signalling results in increased disruption with axons failing to cross in both anterior (open arrow) and posterior commissures (arrowhead). (G) Removal of both Mud and Netrin signalling leads to an enhanced phenotype where midline crossing in the posterior commissure is severely affected and an increased failure of SP1 axons to cross the midline.
Figure 3

Mud acts in neurons and its role in axon guidance is independent of Pins

(A) Mud has previously been identified to function in a partner of inscuteable (Pins)-dependent pathway within neuroblasts. In common with loss of mud, absence of pins does
not lead to significant axon guidance deficits as revealed by the BP102 antibody. Embryos deficient for *mud* and *pins* also show no outgrowth defects. Absence of *pins* does not enhance the axon guidance defects associated with loss of *frazzled* suggesting Mud acts in a Pins-independent pathway to enhance the axon guidance defects caused by a loss of Netrin signalling. (B) Mud overexpression in eagle neurons causes a reduction of midline crossing by the EG neurons which cross through the anterior commissure (AC) revealing mud can influence guidance of neurons. (C) VenusGFP-tagged Mud protein driven by the *mud* promoter is expressed widely within the central nervous system at stage 13 and becomes restricted to subsets of neurons and glia by stage 16. (D) Mud has been found to act downstream from Frizzled. Loss of *frizzled* has little impact on axon outgrowth at the midline as revealed by BP102 yet the double mutant *netAB;fz* is as severe as *Df(1)KA9* or *mud;fra*. This suggests Mud and Frizzled may act in the same pathway which is supported by the fact that a *mud;fz* mutant resembles the phenotype in *mud* or *fz* single mutants.
Tables

Table 1 – Quantification of commissural phenotypes

| BP102 genotype | anterior commissure | posterior commissure | n = |
|----------------|---------------------|----------------------|-----|
|                | normal (%) | thin (%) | absent (%) | normal (%) | thin (%) | absent (%) |
| Df(1)NP5       | 42        | 55       | 3          | 9          | 57       | 34         | 101 |
| Df(1)KA9       | 10        | 56       | 34         | 8          | 41       | 51         | 107 |
| mud³            | 90        | 10       | 0          | 75         | 25       | 0          | 107 |
| fra⁸⁵⁷          | 43        | 29       | 28         | 14         | 56       | 30         | 100 |
| pins⁶²         | 89        | 11       | 0          | 81         | 19       | 0          | 178 |
| fra⁸⁵⁷;pins⁶²   | 31        | 62       | 7          | 10         | 78       | 12         | 251 |
| mud³;fra⁸⁵⁷    | 18        | 47       | 35         | 9          | 51       | 40         | 153 |
| Df(1)KA9*mud   | 44        | 55       | 1          | 29         | 65       | 6          | 102 |
| mud³*mud       | 100       | 0        | 0          | 100        | 0        | 0          | 137 |

| Eagle neurons genotype | anterior commissure (EG) | posterior commissure (EW) | n = |
|------------------------|--------------------------|---------------------------|-----|
|                        | normal (%) | thin (%) | absent (%) | normal (%) | thin (%) | absent (%) |
| Df(1)NP5               | 17        | 63       | 20         | 20         | 43       | 37         | 90  |
| Df(1)KA9               | 11        | 59       | 30         | 5          | 17       | 78         | 83  |

| SP1 neurons genotype | normal crossing (%) | defective crossing (%) | n = |
|----------------------|---------------------|------------------------|-----|
| wild-type            | 100                 | 0                      | 200 |
| mud³                 | 92                  | 8                      | 190 |
| Df(1)NP5             | 79                  | 21                     | 200 |
| Df(1)KA9             | 43                  | 57                     | 190 |

Embryos stained with BP102 were quantified by characterising the thickness of the anterior and posterior commissures within each segment as normal, thin or absent. Thin commissures are those where the commissure is reduced in width to 70% or less of their normal size. The formation of the commissural pathways as revealed by the EgGAL4 driver driving expression of VenusYFP was quantified by characterising the thickness of the anterior and posterior commissures within each segment as normal, thin or absent. Thin commissures are those where the commissure is reduced in width to 70% or less of their normal size. The posterior commissure phenotype of Df(1)KA9 is significantly different to that in Df(1)NP5 (chi-squared test, p<0.0001). Embryos stained with anti-Connectin were quantified by counting the number of hemisegments where the identified SP1 neuron is able to extend across the midline. The SP1 neuron phenotypes are significantly different (one way ANOVA with Bonferroni correction, p<0.0001).
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