



Identification of *cis*-Regulatory Region Controlling Semaphorin-1a Expression in the *Drosophila* Embryonic Nervous System

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The *Drosophila* transmembrane semaphorin Sema-1a mediates forward and reverse signaling that plays an essential role in motor and central nervous system (CNS) axon pathfinding during embryonic neural development. Previous immunohistochemical analysis revealed that Sema-1a is expressed on most commissural and longitudinal axons in the CNS and five motor nerve branches in the peripheral nervous system (PNS). However, Sema-1a-mediated axon guidance function contributes significantly to both intersegmental nerve b (ISNb) and segmental nerve a (SNa), and slightly to ISNd and SNC, but not to ISN motor axon pathfinding. Here, we uncover three *cis*-regulatory elements (CREs), *R34A03*, *R32H10*, and *R33F06*, that robustly drove reporter expression in a large subset of neurons in the CNS. In the transgenic lines *R34A03* and *R32H10* reporter expression was consistently observed on both ISNb and SNa nerve branches, whereas in the line *R33F06* reporter expression was irregularly detected on ISNb or SNa nerve branches in small subsets of abdominal hemisegments. Through complementation test with a *Sema-1a* loss-of-function allele, we found that neuronal expression of Sema-1a driven by each of *R34A03* and *R32H10* restores robustly the CNS and PNS motor axon guidance defects observed in *Sema-1a* homozygous mutants. However,

when wild-type Sema-1a is expressed by *R33F06* in *Sema-1a* mutants, the *Sema-1a* PNS axon guidance phenotypes are partially rescued while the *Sema-1a* CNS axon guidance defects are completely rescued. These results suggest that in a redundant manner, the CREs, *R34A03*, *R32H10*, and *R33F06* govern the Sema-1a expression required for the axon guidance function of *Sema-1a* during embryonic neural development.

Keywords: axon guidance, *cis*-regulatory element, *Drosophila*, motor neurons, semaphorin-1a

INTRODUCTION

The *Drosophila* transmembrane semaphorin-1a (Sema-1a) belongs to the Sema protein family, which is a major group of axon guidance molecules (Dickson, 2002; Pasterkamp, 2012). During embryonic neural development, Sema-1a functions not only as a ligand for PlexA (“forward signaling”), but also as a receptor for unknown ligands (“reverse signaling”) (Jeong et al., 2012; Yu et al., 1998). In the peripheral nervous system (PNS), bidirectional signaling of Sema-1a

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plays an essential role in motor axon pathfinding through mediating axon-axon repulsion (Jeong et al., 2012; 2017). In the central nervous system (CNS), however, *Sema-1a*-mediated forward signaling largely contributes to pathfinding of the outermost FasII-positive axons (Jeong et al., 2012), whereas *Sema-1a*-mediated reverse signaling through an attractive guidance mechanism is required for midline crossing of commissural neurons (Hernandez-Fleming et al., 2017). Furthermore, the receptor function of *Sema-1a* also mediates diverse cellular responses in different areas of the *Drosophila* nervous system, including the giant fiber, visual, and olfactory systems (Cafferty et al., 2006; Godenschwege et al., 2002; Komiya et al., 2007; Pecot et al., 2013; Sweeney et al., 2011). Defining these cell-type specific and context-dependent functions of *Sema-1a* will provide a basis for the understanding of mechanisms underlying precise and complex neural circuit formation. To this end, identification of neurons that express native *Sema-1a* proteins should be an important prerequisite for defining the cell-type specific functions of *Sema-1a*. Previous *in situ* hybridization analysis showed that *Sema-1a* mRNA is robustly detected in a large subset of neurons in the embryonic CNS (Kolodkin et al., 1993). In line with this finding, immunohistochemical staining results showed that *Sema-1a* expression is mainly found on most commissural and longitudinal axons in the CNS and on five motor nerve branches (ISN, ISNb, ISNd, SNa, and SNc) in the PNS (Yu et al., 1998). However, these two approaches are not suitable for investigating the cellular function of *Sema-1a*. For the study of cell-type specific neuronal functions of *Sema-1a*, it should instead be helpful to identify *cis*-regulatory elements (CREs) that govern the native *Sema-1a* expression patterns, and also to have GAL4 lines whose expression is driven by the *Sema-1a* CREs.

Here, we identified three GAL4 lines, *R34A03-GAL4*, *R32H10-GAL4*, and *R33F06-GAL4* that contain the intronic DNA fragments of *Sema-1a* that govern reporter expression on motor nerve branches as well as in a large subset of neurons in the CNS. We observed that highly penetrant guidance phenotypes of *Sema-1a* mutants in the CNS and PNS are significantly rescued by expression of a wild-type *Sema-1a* transgene driven by each of these GAL4 lines. These complementation results suggest that *R34A03*, *R32H10*, and *R33F06* DNA elements redundantly serve as CREs generating the native *Sema-1a* expression patterns during embryonic neural development.

MATERIALS AND METHODS

Drosophila stocks

The *white* mutant allele, *w*¹¹¹⁸, was used as a wild-type control. The following GAL4 lines were obtained from the Bloomington Stock Center: *R90A09-GAL4* (#46907), *R32H10-GAL4* (#45212), *R33G04-GAL4* (#48116), *R34E08-GAL4* (#45221), *R34A12-GAL4* (#45217), *R34D11-GAL4* (#45220), *R33G05-GAL4* (#47346), *R34B01-GAL4* (#45605), *R33C07-GAL4* (#46194), *R33G11-GAL4* (#45964), *R33F11-GAL4* (#47545), *R34A03-GAL4* (#48120), *R33F06-GAL4* (#48115), *R33G02-GAL4* (#47546), *R32G03-GAL4* (#45591), and *R33B07-GAL4* (#45214). All other fly stocks were described previously: *Sema-1a*^{PI} (Yu et al., 1998); *UAS_β-Sema-1a* (Jeong et al., 2012); *UAS-TaumycEGFP* (Callahan et al., 1998); *Sca-GAL4* (Klaes et al., 1994).

Sema-1a^{PI} (Yu et al., 1998); *UAS_β-Sema-1a* (Jeong et al., 2012); *UAS-TaumycEGFP* (Callahan et al., 1998); *Sca-GAL4* (Klaes et al., 1994).

Immunohistochemistry

To precisely examine the expression pattern of each GAL4 line in the PNS and CNS, each GAL4 strain was crossed to *UAS-TaumycGFP* flies. The resulting embryos were immunostained with anti-myc (9E10; Sigma, USA) or anti-GFP (Invitrogen, USA) antibodies and the peroxidase-3,3'-diaminobenzidine reaction (Jeong et al., 2012). To visualize the axonal projection patterns of motor neurons and a small subset of CNS neurons during embryonic neural development, we did immunostaining using anti-FasII antibody, embryo dissection, and imaging as described previously (Jeong, 2017).

Phenotypic characterization

The filleted preparation method was used to better visualize and characterize FasII-positive axon guidance phenotypes observed in the CNS and PNS (Jeong, 2017). The detailed assessment of CNS and motor axon guidance defects were performed as described previously (Van Vector et al., 1993; Yu et al., 1998). The chi-square (χ^2) statistic with a 2 × 2 contingency table was used to assess independence between two categorical variables.

RESULTS

Expression patterns of the GAL4 lines that are driven by flanking and intronic DNA fragments of *Sema-1a*

To identify CREs that are required to direct *Sema-1a* expression during embryonic neural development, we took advantage of a collection of GAL4 lines driven by flanking and intronic DNA fragments of *Sema-1a* (Supplementary Fig. S1). These GAL4 lines were generated by inserting each GAL4 construct into the same genomic location to minimize position effects (Pfeiffer et al., 2008). By crossing to *UAS-GFP-NLS*, which encodes a nuclear localized GFP protein, the expression patterns of most GAL4 lines were characterized in stage 16 embryos and are available online (Manning et al., 2012). These expression patterns visualized by nuclear localization of GFP reporter protein are clearly valuable for identifying tissue-specific expression patterns, quantification of cells expressing the reporters, and neuronal morphology (Manning et al., 2012). However, in the embryonic CNS, it is hard to identify the cell bodies of motor neurons that extend their axons out of the CNS to form the five motor nerve branches. Therefore, based on their available expression patterns and genomic organization of the *Sema-1a* gene that contains one *ncRNA*, one 297/327 transposable element, and two different *tRNA* genes in the intronic region, we selected a collection of 16 GAL4 lines for further analysis (Fig. 1). To visualize the axonal expression pattern of each GAL4 line in the PNS as well as in the CNS, we crossed each line to a *UAS-TaumycGFP* reporter, which is primarily localized to the axonal compartment, and then stained the resulting embryos with anti-myc or anti-GFP antibodies (Jeong et al., 2012). We obtained reporter expression data derived from a collection of 16 GAL4 lines (Fig. 2, Supplementary Fig. S2).

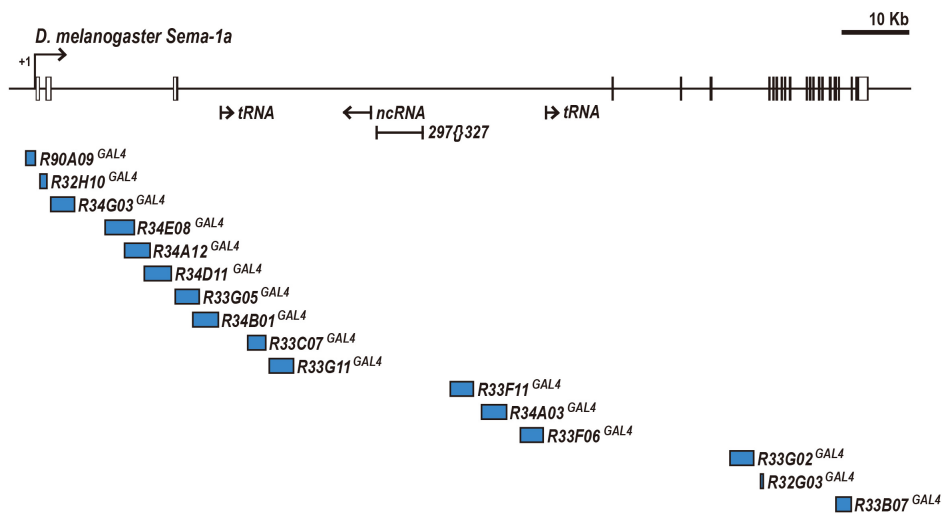


Fig. 1. Genomic organization of the *Sema-1a* gene and the positions of the genomic DNA fragments tested for their GAL4 expressions. The coding and noncoding exons of *Sema-1a* are represented by the closed and open boxes, respectively. Two *tRNA* genes, one *ncRNA*, and one *297Q327* transposable element are located within the third intron of *Sema-1a*. Arrows denote the direction of transcription of genes. Blue boxes displayed below the map indicate the length and position of the genomic DNA fragments that were used for generating *Sema-1a* GAL4 drivers.

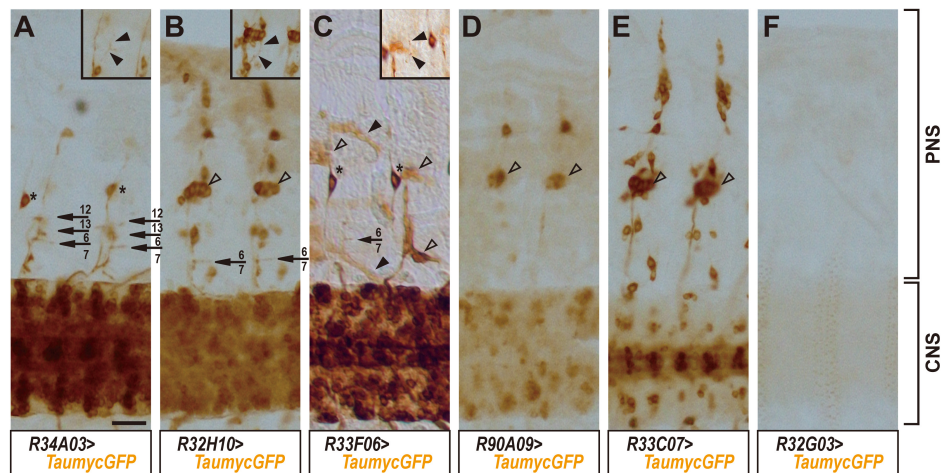


Fig. 2. Expression patterns of a selected set of *Sema-1a* GAL4 lines. (A-F) Stage 16 embryos were stained with anti-myc or anti-GFP antibodies and then filled embryos were prepared to visualize reporter expression patterns driven by GAL4 drivers. In the Figs. 2A-2C, numbers indicate the location of the ventrolateral muscles 6, 7, 12, and 13, and each inset represents the SNa nerve branch in a different focal plane. Anterior is left and dorsal is up. Scale bar = 20 μ m. (A) Line *R34A03-GAL4* robustly drives reporter expression in a large subset of cells in the CNS. In the PNS, reporter expression is detected on both ISNb (arrows) and SNa (arrowheads in the inset) nerve branches and also on the lateral bipolar dendrite neurons (asterisks). (B) Line *R32H10-GAL4* moderately drives reporter expression in a large subset of cells in the CNS. In the PNS, reporter expression is observed on both ISNb (arrows) and SNa (arrowheads in the inset) nerve branches and sensory nerve fibers (open arrowheads). (C) Line *R33F06-GAL4* drives strong reporter expression in a large subset of cells in the CNS and irregular expression on ISNb (arrow) or SNa (arrowheads in the inset) nerve branches in small subsets of hemisegments in the PNS. In addition, reporter expression is also detected consistently in lateral bipolar dendrite neurons (asterisks) and irregularly in the body wall, including some muscle fibers (arrowheads) and unidentified cells (open arrowheads). (D) Line *R90A09-GAL4* sparsely drives reporter expression in a small subset of cells in the CNS. In the PNS, a small subset of sensory cells expresses reporter proteins (open arrowheads). (E) Line *R33C07-GAL4* drives reporter expression in a small subset of cells in the CNS and sensory nerve fibers (open arrowheads) in the PNS. (F) Line *R32G03-GAL4* barely drives reporter expression in the CNS and PNS.

Among them, we found that *R34A03-GAL4* and *R32H10-GAL4* drove reporter expression in ISNb, SNa, and ISN nerve branches, although they also showed expression in lateral bipolar dendrite neurons (LBDs) or sensory neurons in which endogenous Sema-1a proteins were not detected (Figs. 2A and 2B) (Yu et al., 1998). Furthermore, in the *R33F06-GAL4* line we observed irregular reporter expression on ISNb or SNa nerve branches in some abdominal hemisegments, consistent expression in LBDs, and also irregular expression in some muscles and unidentified cells (Fig. 2C). In addition, these three GAL4 lines drove robust reporter expression in large subsets of cells in the CNS (Figs. 2A-2C). Given that endogenous Sema-1a is mainly expressed on most commissural and longitudinal axons in the CNS and five motor nerve branches in the PNS (Yu et al., 1998), these findings may indicate that *R34A03-GAL4*, *R32H10-GAL4*, and *R33F06-GAL4* contain CREs that are responsible for neuronal Sema-1a expression in the PNS and CNS. We also found that the other GAL4 lines barely drove reporter expression in ISNb and SNa nerve branches (Figs. 2D-2F, Supplementary Fig. S2). However,

these GAL4 lines showed expression in subsets of cells in the CNS except *R32G03-GAL4*, in which reporter expression was hardly detected (Figs. 2C-2F, Supplementary Fig. S2). As described previously (Manning et al., 2012), in these GAL4 lines we also observed reporter expression in the body wall, including muscles, sensory neurons, and other cell types (Figs. 2D-2F, Supplementary Fig. S2). Furthermore, we found that *R34A12-GAL4* and *R33F11-GAL4* showed two different expression patterns, probably because of either multiple insertions of a transgene or line contamination (Supplementary Figs. S2C, S2D, S2H, and S2I).

Rescue of motor axon guidance phenotypes observed in *Sema-1a* mutants with a selected GAL4 lines

To find out whether *R34A03-GAL4*, *R32H10-GAL4*, and *R33F06-GAL4* drive the Sema-1a expression required for the axon guidance function of *Sema-1a* during embryonic neural development, we expressed a *Sema-1a* transgene using either GAL4 line in *Sema-1a^{PI}* loss-of-function mutants. In wild-type embryos, ISNb motor neurons, whose cell bod-

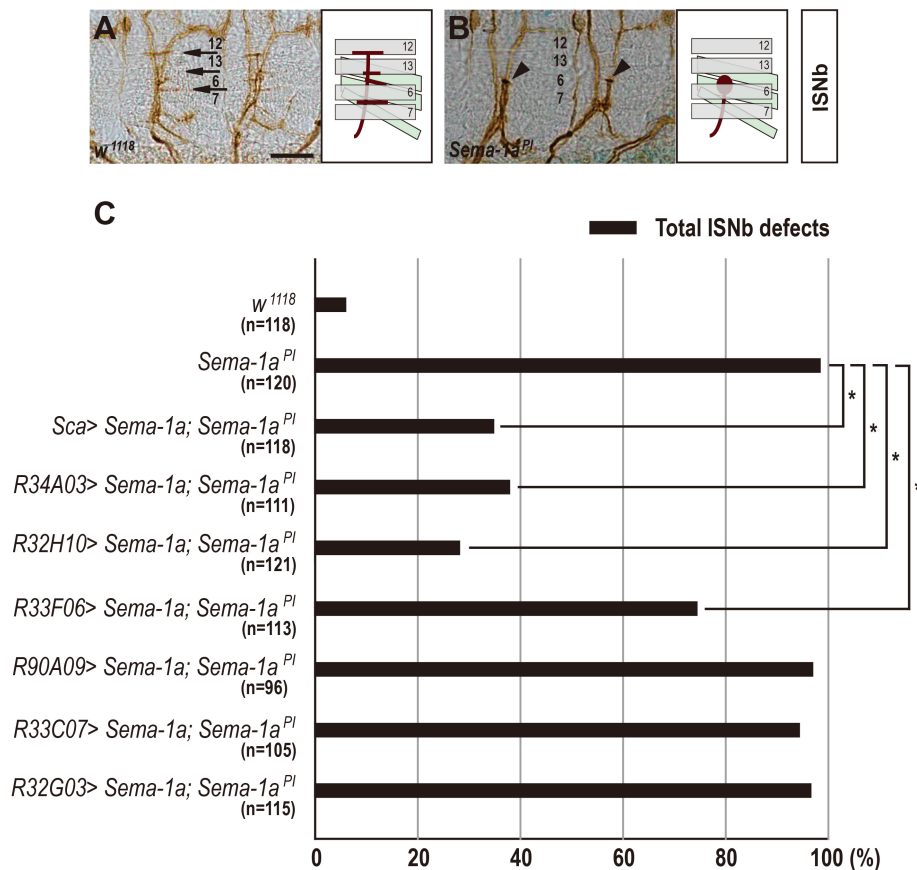


Fig. 3. Rescue of *Sema-1a* ISNb axon guidance defects with GAL4 lines. (A and B) Late stage 16 embryos were stained with anti-FasII antibodies and then filled embryos were prepared to visualize axonal projection patterns of ISNb motor neurons. Anterior is left and dorsal is up. Schematic drawings for the muscle innervation patterns of ISNb motor neurons are represented in each panel. Scale bar = 20 μ m. (A) In wild-type embryos, ISNb axons selectively defasciculate at specific choice points (arrows), innervating their target muscles 6, 7, 13, and 12. (B) In *Sema-1a* mutant embryos, ISNb axons frequently fail to defasciculate at specific choice points, resulting in innervation defects (arrowheads). (C) Percentages of total ISNb defects (closed bars) in embryos with indicated genotypes ($*P < 0.001$, χ^2 test). n = number of hemisegments scored for total ISNb defects.

ies reside in the ventral nerve cord, extend their axons in a fascicle toward the ventrolateral muscle region and finally innervate muscles 6, 7, 12, 13, 14, 28, and 30 (Fig. 3A) (Landgraf and Thor, 2006; Van Vactor et al., 1993). Normally, SNa motor axons innervate lateral muscles 5, 8, 21, 22, 23, and 24 (Fig. 4A) (Landgraf and Thor, 2006; Van Vactor et al., 1993). In both ISNb and SNa pathways, selective defasciculation of motor axons at specific choice points is required for correct innervation of their target muscles (Figs. 3A and 4A) (Jeong, 2017). *Sema-1a^{Pf}* mutants exhibited highly penetrant guidance defects in the ISNb and SNa pathways (98.3% and 76.1% of hemisegments, respectively; Figs. 3B, 3C, 4B, and 4C) (Yu et al., 1998). This can be explained by the finding that bidirectional signaling of Sema-1a is required for selective defasciculation of ISNb and SNa motor axons at specific choice points (Jeong et al., 2012; 2017). Interestingly, neuronal expression of *Sema-1a* using either *R34A03-GAL4* or *R32H10-GAL4* robustly restored the ISNb (37.8%

and 28.1% of hemisegments, respectively; Fig. 3C) and SNa guidance defects (30.4% and 28.0% of hemisegments, respectively; Fig. 4C) observed in *Sema-1a^{Pf}* mutants, consistent with their expression that recapitulates the native Sema-1a expression pattern in the PNS (Figs. 2A and 2B) (Yu et al., 1998). A similar rescue effect was also observed in *Sema-1a^{Pf}* mutant embryos expressing *Sema-1a* under the control of *Sca-GAL4*, which drives expression in all neuroblasts and their progeny (34.8% and 23.9% of hemisegments, respectively; Figs. 3C and 4C) (Klaes et al., 1994). In addition, expression of *Sema-1a* using *R33F06-GAL4* partially restored both the ISNb and the SNa guidance defects of *Sema-1a^{Pf}* mutants (74.3% and 44.3% of hemisegments, respectively; Figs. 3C and 4C), reflecting the fact that irregular and weak reporter expression was detected on ISNb or SNa nerve branches (Fig. 2C). These data strongly suggest that DNA fragments *R34A03*, *R32H10*, and *R33F06* govern redundantly expression of endogenous Sema-1a responsible for its motor axon

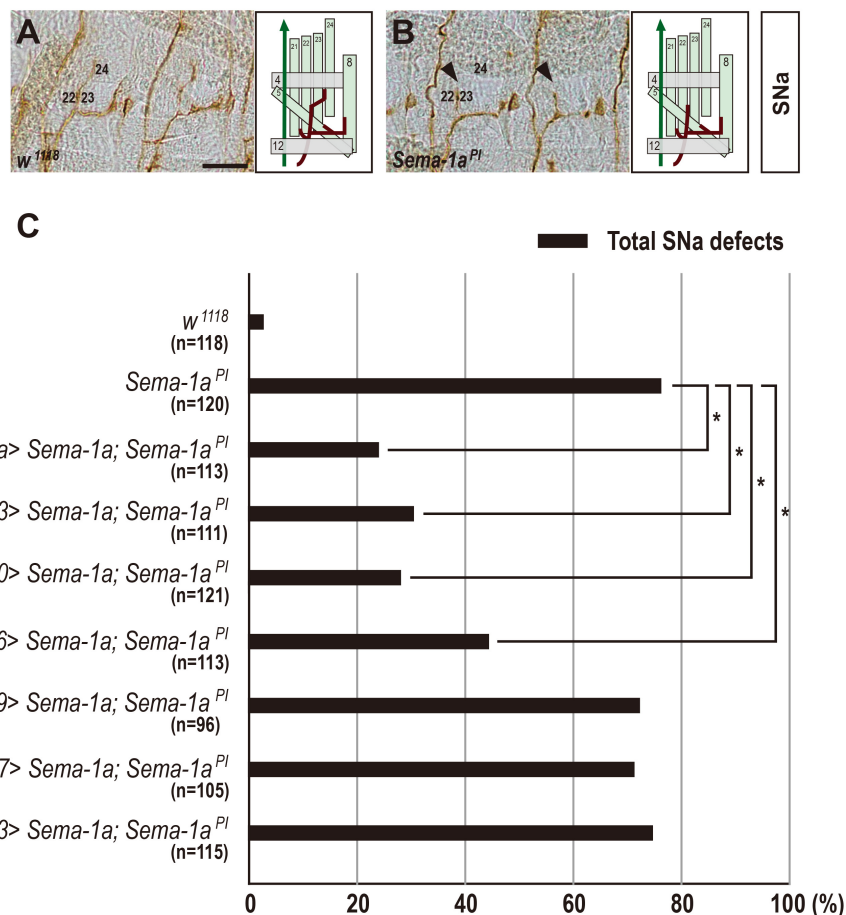


Fig. 4. Rescue of *Sema-1a* SNa axon guidance defects with GAL4 lines. (A and B) Late stage 16 embryos were stained with anti-FasII antibodies and then filleted embryos were prepared to visualize axonal projection patterns of SNa motor neurons. Anterior is left and dorsal is up. Schematic drawings for the muscle innervation patterns of SNa motor neurons are represented in each panel. Scale bar = 20 μ m. (A) In wild-type embryos, SNa axons normally defasciculate to innervate target muscles 5, 8, 21, 22, 23, and 24. (B) In *Sema-1a^{Pf}* mutant embryos, ISNb axons frequently fail to defasciculate at a specific choice point located between muscles 22 and 23, showing lack of innervation in muscle 24 (arrowheads). (C) Percentages of total SNa defects (closed bars) in embryos with indicated genotypes (**P* < 0.001, χ^2 test). n = number of hemisegments scored for total SNa defects.

guidance function. Furthermore, we also examined the ability of other GAL4 lines, such as *R90A09-GAL4*, *R33C07-GAL4*, and *R32G03-GAL4*, to rescue the PNS guidance defects of *Sema-1a* mutants. As expected, any of these GAL4 lines did not significantly rescue the ISNb as well as SNa guidance defects observed in *Sema-1a* mutants (Figs. 3C and 4C). These complementation results are concordant with our observation that reporter expression driven by these GAL4 lines was barely detected in both ISNb and SNa nerve branches (Figs. 2D-2F).

Rescue of CNS axon guidance phenotypes observed in *Sema-1a* mutants with a selected GAL4 lines

Since *R34A03-GAL4*, *R32H10-GAL4*, and *R33F06-GAL4* drove robust expression in large subsets of cells in the CNS (Figs. 2A and 2B), we next assessed these GAL4 lines for their ability to rescue CNS axon guidance defects observed in *Sema-1a* mutants. Immunohistochemistry with anti-FasII antibody visualizes three longitudinal axon fascicles on each side

of the midline in the wild-type CNS (Fig. 5A) (Grenningloh et al., 1991). Frequently observed disruptions in the outermost FasII-positive fascicles of *Sema-1a* mutants (70.7% of hemisegments; Figs. 5B and 5C) (Yu et al., 1998) were robustly rescued by neuronal expression of *Sema-1a* using either *R34A03-GAL4* (2.3% of hemisegments; Fig. 5C), *R32H10-GAL4* (7.7% of hemisegments; Fig. 5C), or *R33F06-GAL4* (0.0% of hemisegments; Fig. 5C). Along with the fact that neuronal expression of *Sema-1a* using *Sca-GAL4* restored the CNS guidance defects of *Sema-1a* mutants to wild-type levels (Fig. 5C), these observations demonstrate that DNA fragments *R34A03*, *R32H10*, and *R33F06* control redundantly expression of *Sema-1a* required for the axon guidance function of *Sema-1a* in the embryonic CNS. We also examined the same set of GAL4 lines, *R90A09-GAL4*, *R33C07-GAL4*, and *R32G03-GAL4*, which were used in rescue of the PNS guidance defects (Figs. 2D-2F), for their ability to recover the CNS guidance phenotypes of *Sema-1a* mutants and found that any of these GAL4 lines did not significantly rescue the

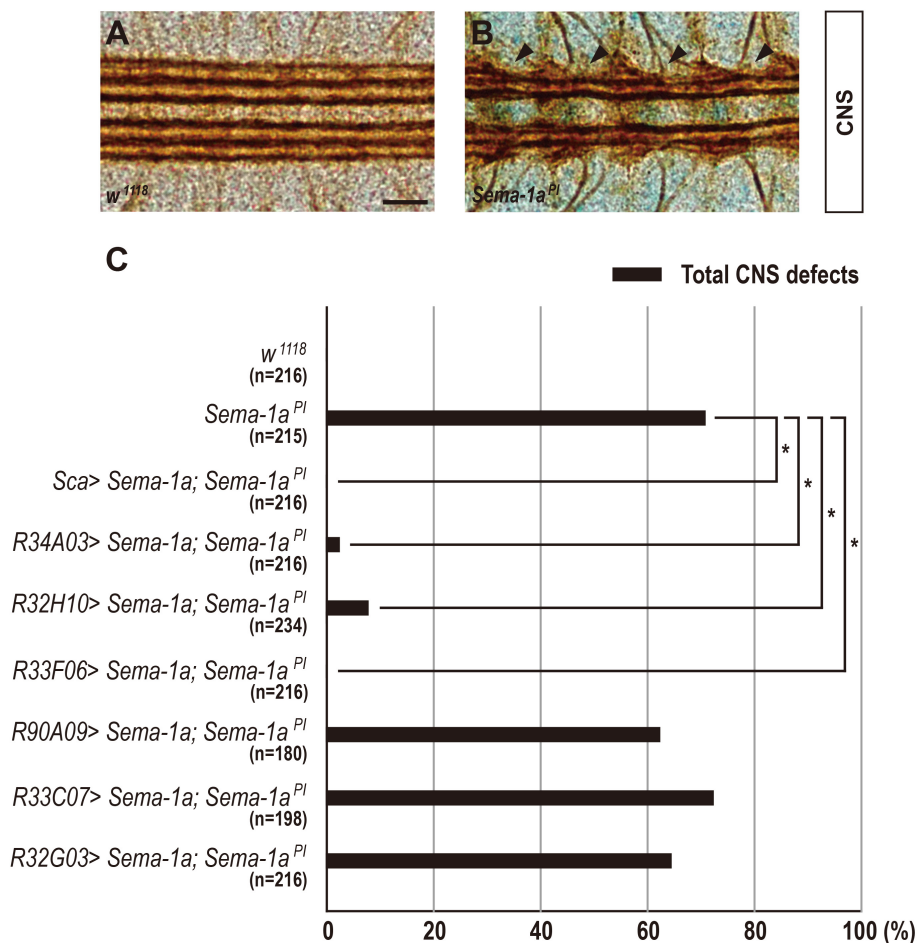


Fig. 5. Rescue of *Sema-1a* CNS guidance defects with GAL4 lines. (A and B) Late stage 16 embryos were stained with anti-FasII antibodies and then filleted embryos were prepared to visualize the projection patterns of longitudinal axon fascicles in the CNS. Anterior is left. Scale bar = 20 μ m. (A) Three parallel axon fascicles on either side of the CNS are observed in wild-type embryos. (B) Frequent disruptions in the outermost fascicles are found (arrowheads) in *Sema-1a* mutant embryos. (C) Percentages of total CNS defects (closed bars) in embryos with indicated genotypes (* $P < 0.001$, χ^2 test). n = number of hemisegments scored for total CNS defects.

CNS guidance defects (Fig. 5C). Taken together, our results suggest that three DNA fragments, *R34A03*, *R32H10*, and *R33F06*, serve as CREs that govern *Sema-1a* expression required for the axon guidance function of *Sema-1a* in the CNS as well as the PNS.

DISCUSSION

Here, we provide evidence that endogenous *Sema-1a* expression is controlled by multiple CREs *R34A03*, *R32H10*, and *R33F06* in a redundant manner during embryonic neural development. First, both ISNb and SNa motor axon guidance defects seen in *Sema-1a* null mutants are robustly rescued by the expression of wild-type *Sema-1a* under the control of either *R34A03-GAL4* or *R32H10-GAL4*. Consistently, these CREs drove strong reporter expression on both ISNb and SNa nerve branches. We also found that the PNS axon guidance phenotypes of *Sema-1a* mutants are partially rescued when *Sema-1a* expression is reintroduced under the control of *R33F06-GAL4*. This could be explained by the fact that in an irregular manner, we detected low levels of reporter expression on ISNb or SNa nerve branches in transgenic reporter assays using *R33F06-GAL4*. Moreover, we previously observed mild but significant motor axon guidance defects in embryos transheterozygous for the *Sema-1a* null allele, which would be expected to have an approximately 50% reduction in the expression of *Sema-1a*, indicating haplo-insufficiency of *Sema-1a* (Jeong et al., 2012; 2017). Taken together, our findings demonstrate that *Sema-1a* expression on both ISNb and SNa nerve branches is regulated by multiple redundant CREs such as *R34A03*, *R32H10*, and *R33F06*. Second, *Sema-1a* null mutation leads to frequent disruptions in the outermost FasII-positive fascicles in the CNS (Figs. 5B and 5C), indicative of a critical role in CNS axon guidance. This CNS guidance phenotype was rescued to wild-type levels in *Sema-1a* mutant embryos expressing *Sema-1a* under the control of *R33F06-GAL4*. We also found that the CNS axon guidance defects of *Sema-1a* robustly but incompletely rescued by either *R34A03-GAL4* or *R32H10-GAL4*. These results strongly suggest that *R34A03*, *R32H10*, and *R33F06* serve as CREs to redundantly govern endogenous *Sema-1a* expression required for the FasII-positive axon guidance function of *Sema-1a* in the embryonic CNS.

It has been reported that there are usually four developmental enhancer elements per protein-coding gene in the *Drosophila* genome on average during embryonic development (Kvon et al., 2014). A growing body of evidence suggests that functional redundancy of multiple CREs is a common feature of gene-regulatory networks in vertebrate and invertebrate development (Frankel et al., 2010; Montavon et al., 2011; Osterwalder et al., 2018; Perry et al., 2010) and further demonstrates that functional redundancy contributes to precise spatiotemporal patterns and robustness of gene expression in response to environmental or genetic variation (Barolo, 2012; Cannavò et al., 2016). In addition, changes within multiple CREs with overlapping expression activities have been proposed to provide better opportunities for evolutionary innovations especially for highly pleiotropic genes (Barolo, 2012; Prud'homme et al., 2007). Given that the

pleiotropic *Sema-1a* gene plays a critical role in both motor neuron and CNS axon guidance during neural development (Yu et al., 1998), the multiple redundant CREs of *Sema-1a* that we have identified here should collaborate to precisely regulate the spatiotemporal patterns and levels of endogenous *Sema-1a*. Furthermore, *R34A03-GAL4* and *R33F06-GAL4* lines have been reported to drive reporter expression in some overlapping regions of the adult brain including the antenna lobe (AL), lateral horn (LH), and mushroom body (MB) (Jenett et al., 2012; image data are found at Janelia's FlyLight site). Interestingly, *Sema-1a* is required for axon targeting of olfactory receptor neurons and dendritic targeting of olfactory projection neurons (PNs) in the AL as well as axon targeting of PNs to the appropriate areas of the MB and the LH (Komiyama et al., 2007; Sweeney et al., 2007). These suggest that *R34A03* and *R33F06* also have enhancer redundancy for governing the expression of *Sema-1a* in the adult brain. In contrast, *R32H10-GAL4* line displayed broad reporter expression in the adult brain and ventral nerve cord, but may not be in the brain regions such as AL, LH, MB (Jenett et al., 2012; image data are found at Janelia's FlyLight site). Taken together, these results demonstrate that the CREs, *R34A03*, *R32H10*, and *R33F06*, show similar, overlapping, or unique enhancer activities for controlling *Sema-1a* expression in the embryonic and adult nervous systems.

Since transmembrane *Sema-1a* functions as a ligand (forward signaling) and/or as a receptor (reverse signaling) to mediate axon-axon repulsion at specific choice points in the PNS (Jeong et al., 2012; 2017; Yu et al., 1998), one important question related to *Sema-1a* expression is which motoneurons express native *Sema-1a* proteins during embryonic development. Interestingly, we found that the reporter expression patterns of *R34A03-GAL4* on ISNb and SNa nerve branches (Fig. 2A) are very similar to the axonal projection patterns of ISNb and SNa stained with anti-FasII antibodies (Fig. 3A). These observations may indicate that *Sema-1a* is expressed in most ISNb and SNa motor neurons. There is one caveat to this interpretation because the reporter expression of *R34A03-GAL4* is also observed in lateral bipolar dendrite neurons where endogenous *Sema-1a* is barely detected (Fig. 2A) (Yu et al., 1998). Furthermore, *R32H10-GAL4* and *R33F06-GAL4* drove reporter expression in unanticipated sensory neurons and muscle fibers, respectively (Figs. 2B and 2C). These unanticipated reporter expression patterns of individual transgenic CREs might result from the absence of repressive element(s) located within neighboring CREs, which restrict aberrant CRE activity in the genome (Dunipace et al., 2011; Perry et al., 2011). Another interesting question is how *Sema-1a*-mediated selective repulsion between motor axons can be achieved if most motor neurons in the ISNb and SNa nerve branches express native *Sema-1a* proteins. One possible mechanism for the selective activation of *Sema-1a* repulsive signaling could be through the differential recruitment of co-receptors and/or intracellular signaling mediators (Jongbloets and Pasterkamp, 2014; Pasterkamp, 2012).

Note: Supplementary information is available on the *Molecules and Cells* website (www.molcells.org).

Disclosure

The authors have no potential conflicts of interest to disclose.

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