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Characterization of *plexinA* and two distinct *semaphorin1a* transcripts in the developing and adult cricket *Gryllus bimaculatus*

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Abstract

Guidance cues act during development to guide growth cones to their proper targets in both the central and peripheral nervous systems. Experiments in many species indicate that guidance molecules also play important roles after development, though less is understood about their functions in the adult. The Semaphorin family of guidance cues, signaling through Plexin receptors, influences the development of both axons and dendrites in invertebrates. Semaphorin functions have been extensively explored in *Drosophila melanogaster* and some other Dipteran species, but little is known about their function in hemimetabolous insects. Here, we characterize *sema1a* and *plexA* in the cricket *Gryllus bimaculatus*. In fact, we found two distinct predicted Sema1a proteins in this species, Sema1a.1 and Sema1a.2, which shared only 48% identity at the amino acid level. We include a phylogenetic analysis that predicted that many other insect species, both holometabolous and hemimetabolous, express two Sema1a proteins as well. Finally, we used in situ hybridization to show that *sema1a.1* and *sema1a.2* expression patterns were spatially distinct in the embryo, and both roughly overlap with *plexA*. All three transcripts were also expressed in the adult brain, mainly in the mushroom bodies, though *sema1a.2* was expressed most robustly. *sema1a.2* was also expressed strongly in the adult thoracic ganglia while *sema1a.1* was only weakly expressed and *plexA* was undetectable.

KEYWORDS
axon guidance cues, dendritic guidance, hemimetabolous, phylogeny, plasticity, RRID: AB_2734716, RRID:SCR_002077, RRID:SCR_002760, RRID:SCR_004801, RRID: SCR_006724, RRID:SCR_008406, RRID:SCR_010519, RRID:SCR_013672, RRID: SCR_014199, RRID:SCR_014935, RRID:SCR_017105, sequence analysis

1 | INTRODUCTION

The establishment and maintenance of neuronal circuits is critical to development and survival. Various guidance cues help axons make appropriate pathway decisions as growth cones navigate toward their targets in order to form functional synapses. The Semaphorins (Semas) are one family of guidance cues that, along with their Plexin receptors, are involved in multiple stages of this sequential process. Semas can act as repellents or attractants during development in both vertebrates (Polleux, Morrow, & Ghosh, 2000) and invertebrates (Wu et al., 2011). Semas act to guide axons to their target regions in vertebrates (Falk et al., 2005; Renzi, Wexler, & Raper, 2000) and insects (Isbister, Tsai, Wong, Kolodkin, & O'Connor, 1999; Roh, Yang, & Jeong, 2016), and they also help guide and shape maturing

dendrites in rodents (Fenstermaker, Chen, Ghosh, & Yuste, 2004; Morita et al., 2006; Tran et al., 2009) and in flies (Hernandez-Fleming, Rohrbach, & Bashaw, 2017; Jan & Jan, 2003; Kim & Chiba, 2004; Syed, Gowda, Reddy, Reichert, & VijayRaghavan, 2016). In addition, Sema signaling is important for synapse formation during development of vertebrate and invertebrate nervous systems (Murphey, 2003; Nakamura et al., 2009). Sema signaling has also been found to be important for circuit maintenance in mature vertebrate nervous systems (Ng et al., 2013; Sahay et al., 2005).

The Semaphorin family members are grouped into eight different classes. Invertebrates express transmembrane (Class 1 and Class 5) and a secreted form (Class 2), while Classes 3–7 are expressed in vertebrates. An eighth class is expressed in viruses (Yazdani & Terman, 2006). All members of this family possess a well-conserved semaphorin domain, that is, approximately 500 amino acids long and contains between 14 and 16 cysteine residues. These cysteine residues are presumably important for the structure of the receptor-binding domain (Yazdani & Terman, 2006).

Invertebrate Semas mainly bind Plexin (Plex) receptors (Ayoob, Terman, & Kolodkin, 2006; Winberg, Noordermeer, & Tamagnone, 1998), which are thought to be ancestrally related to Semas since they contain a sema domain (Winberg et al., 1998). Specifically, Sema1a and Sema1b bind PlexA receptors (Winberg et al., 1998), while Sema2a binds PlexB receptors (Ayoob et al., 2006). Semaphorins can also interact with a variety of co-receptors, including Off-Track (Winberg et al., 2001) and Guanylyl cyclase at 76C (Gyc76C), the latter of which appears to be required for Sema1a-PlexA-mediated repulsion of axons (Ayoob, Yu, Terman, & Kolodkin, 2004). Semas are also capable of bi-directional signaling, indicating that intracellular "reverse" signaling can happen downstream of the transmembrane Semas (Battistini & Tamagnone, 2016).

Semaphorins play an important role in the development of both the peripheral nervous system (PNS) and the central nervous system (CNS) in invertebrates. In fact, the first Sema was discovered in grasshopper (originally called Fas IV; Kolodkin et al., 1992) where it has a well-described role guiding peripheral axons toward the CNS (Isbister et al., 1999; Wong, Wong, & O'connor, 1999; Wong, Yu, & O'connor, 1997). Sema1a and Sema2a are expressed in grasshopper limb buds in complementary bands that together influence the proper directed growth and fasciculation of the tibial pioneer axons (Isbister et al., 1999; Kolodkin et al., 1992; Wong et al., 1999). In *D. melanogaster*, *sema1a* transcripts are expressed in the developing CNS (Kolodkin, Matthes, & Goodman, 1993) and analysis of knockouts indicates Sema1a protein expression is important for proper motor neuron and CNS axon pathfinding (Yu, Araj, Ralls, & Kolodkin, 1998). More recently, knockdown of *sema1a* in *A. aegypti* indicates that it also plays an important role in normal CNS development in the mosquito (Haugen et al., 2011).

The expression patterns of the Semas and Plexins have been described most thoroughly during development, but both continue to be expressed in some CNS regions into adulthood. Semas can be found in the adult CNS in both vertebrates (Duan et al., 2014; Giger, Pasterkamp, Heijnen, Holtmaat, & Verhaagen, 1998) and invertebrates (Maynard, McCarthy, Sheldon, & Horch, 2007), typically in areas of high plasticity such as the hippocampus (Sahay et al., 2005) and the mushroom bodies (Eickhoff & Bicker, 2012; Maynard et al., 2007). Plexins have also been described in the adult CNS in vertebrates (Gutekunst, Stewart, & Gross, 2010; Saha, Ypsilanti, Boutin, Cremer, & Chedotal, 2012; Usui, Taniguchi, Yokomizo, & Shimizu, 2003), and invertebrates (Yoo, 2016) though less is known about Plexin distribution at this stage.

Here, we characterize the sequences and expression patterns of *sema1a* and *plexA* in the cricket, *Gryllus bimaculatus*. The cricket is a hemimetabolous Orthopteran of the Polyneoptera cohort; phylogenetic analysis of insect orders predict that Orthoptera branched basally to the holometabolous insects (Misof et al., 2014). Much of what is known about neuronal development in insects is based on studies of holometabolous insects, *D. melanogaster* most prominently. The cricket, however, has emerged as a molecular developmental model since it sits in a useful phylogenetic location for comparative studies. Few experiments have yet characterized the expression or function of neuronal developmental guidance cues in this species. Here, we describe sequence analysis and expression patterns for *sema1a* and *plexA* in *G. bimaculatus*. We discovered two distinct *sema1a*-type transcripts, which we will refer to as *sema1a.1* and *sema1a.2*. The predicted protein sequences, though more similar to each other than to any other Sema protein, shared only 48% identity at the amino acid level. An in silico search identified pairs of Sema1a-type proteins in a number of other insect species and a phylogenetic analysis indicated that this was likely an ancient gene duplication. In *G. bimaculatus*, *sema1a.1* and *sema1a.2* were expressed in spatially distinct patterns in developing embryos and were present in the adult CNS, including in the mushroom bodies. Our documentation of the expression patterns for *sema1a.1*, *sema1a.2*, and *plexA* in embryos and in adult CNS tissues lays the groundwork for understanding the roles played by these proteins in developing and mature nervous systems.

2 | MATERIALS AND METHODS

2.1 | Animals

A colony of *G. bimaculatus* (originally obtained from Ron Hoy at Cornell University) was maintained on a 12:12 light: dark cycle, at 28°C, and at 60–80% relative humidity. Crickets were given water and cat chow ad libitum.

2.2 | Cloning

Partial *sema1a* sequences were obtained from a draft of the *G. bimaculatus* genome (S. Noji and T. Mito pers. comm.) and from published transcriptomes (Fisher et al., 2018; Zeng et al., 2013). Complete *plexA* sequence was obtained from a published transcriptome (Fisher et al., 2018). Polymerase chain reaction (PCR) on cDNA derived from embryos and adult prothoracic ganglia, followed by Sanger sequencing, was used to confirm these sequences and to obtain complete sequence for any gaps that existed. Sequence data for *sema1a.1* (Accession # MK473371), *sema1a.2* (MF817714.1), and

plexA (MK473372) can be found in GenBank (GenBank, RRID: SCR_002760). The transcriptome data have been previously described (Fisher et al., 2018) and have been deposited with links to BioProject accession number PRJNA376023 in the NCBI BioProject database (NCBI BioProject, RRID:SCR_004801).

2.3 | Sequence analysis

Sanger sequence results were translated, analyzed for conserved domains, and aligned in Geneious 10.2.6 (Geneious, RRID:SCR_010519). Predicted amino acid sequences were aligned to each other and to protein sequences from *Schistocerca americana* (grasshopper) Sema1a (AAA29808.1) and *D. melanogaster* Sema1a (NP_001260265.1). Although much of the functional work on grasshopper Semas was completed in the grasshopper *Schistocerca gregaria* (Isbister et al., 1999; Isbister, Mackenzie, To, & O'Connor, 2003; Wong et al., 1997), no Sema1a sequences are publicly available in this species, so Sema1a from the grasshopper *S. americana* was used for comparison here. Pairwise identities were calculated in Geneious. Transmembrane domains were predicted using TMHMM Server v. 2.0 (TMHMM Server, RRID:SCR_014935; Krogh, Larsson, von Heijne, & Sonnhammer, 2001). A search for the conserved domains that might be present in the different Sema1a.1 splice variants was completed on 8-22-2019 using the NCBI Conserved Domain database (Conserved Domain Database, RRID:SCR_002077). All phylogenetic trees were created in Geneious Prime 2019.0.4 (Geneious, RRID: SCR_010519), and bootstrap statistics, based on 1,000 replicates, were applied. A Sema tanglegram (Figure 3) was created based on comparison of two unrooted trees built with the UPGMA algorithm. Proteins in red on the left were identified using Sema1a.1 as a query while proteins in blue on the right were identified using Sema1a.2 as a query. The PlexA tree (Figure 4) was a rooted tree (with *Caenorhabditis elegans* as the outgroup) created using the Neighbor Joining algorithm. Bootstrap values were included at each node in all trees.

2.4 | Synthesis of digoxigenin-labeled RNA probes

A 499 base pair fragment of the *sema1a.2* coding region was initially amplified using gene specific primers (5′ GCAGTGTTGGAGGATTGTCA 3′ and 5′ CGGAGAACTGA AAGAGGGTCT 3′) and cloned into pGEM-T Easy vectors (Promega, RRID:SCR_006724). The insert was sequenced to confirm identity and directionality. Probe template was amplified using forward and reverse primers against the T7 and SP6 sites and purified by phenol chloroform extraction and ethanol precipitation. *sema2a* probes were synthesized as previously described (Maynard et al., 2007). Briefly, a 756-bp fragment of the *sema2a* coding region was amplified from cDNA with gene-specific primers (5′ ACCATGTACGCGAGTTCTCCT 3′ and 5′ GAGGTAGGTGGCCCAGTT CT 3′), sequenced, and synthesized as described above for *sema1a.2*. In addition, a 499 bp *sema1a.2* coding region sequence (beginning at GCAGTGTTGGAGGATTGTCAT) plus a T7 binding site, a 500 bp *sema1a.1* coding region sequence (beginning at GCTGAGGAATGTGTT AAGAAA) plus a T7 binding site, and a 570 bp *plexA* 3′UTR sequence (starting at TGATGGCGGTCGGCCGGGCCA) plus a T7 binding site were synthesized as gBlocks by Integrated DNA Technologies (IDT, Sekokie, IL). These gBlocks were used as templates for probe synthesis. T7 RNA polymerase (Ambion, Inc., RRID:SCR_008406) was used on all templates to synthesize digoxignin (DIG-11-UTP)-labeled riboprobes. Probes synthesized from vectors and gBlocks gave staining patterns that were not distinguishable from each other.

2.5 | Whole-mount in situ hybridization

Embryos were extracted from eggs that were 2.5–6 days in age. Embryonic stages (ES 6–9.5) were assigned based on published criteria (Donoughe & Extavour, 2016; Niwa et al., 2000), and these stages were also converted to % development (19–49%) based on data from Donoughe and Extavour (2016) to facilitate comparison with grasshopper development. Embryos were removed from eggs and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at room temperature. Thoracic ganglia and brains were dissected from adult *G. bimaculatus* and fixed for 1–2 hr in 4% PFA in PBS, desheathed in PBS, and returned to 4% PFA in PBS overnight at room temperature. Whole-mount in situ hybridization (ISH) was performed as described (Horch, Liu, Mito, Popadic, & Watanabe, 2017). Briefly, tissue was dehydrated in a methanol series, rehydrated, and incubated in 2 μg/ml Proteinase K. Embryos were treated in Proteinase K for 5–10 min, thoracic ganglia for 15 min, and brains for 20 min. Tissue was refixed then hybridized in probe overnight at 60 or 70°C, and was then blocked for 60 min followed by an incubation with a 1:2,500 dilution of Anti-DIG AP Fab fragments (Sigma-Aldrich Cat# 11093274910, RRID: AB_2734716) Lot # 12486522 for 60 min. The color reaction was performed at either room temperature or 28°C in NBT and BCIP. Some of the wash steps in a few of the experiments were completed using a BioLane HTI (Intavis, Cologne, Germany). *sema2a* ISH results have been previously described (Maynard et al., 2007), and *sema2a* probes were included in each experiment for comparison purposes. Approximately, equal numbers of adult male and female brains and ganglia were used, but since no sexually dimorphic expression patterns were observed, results were combined.

2.6 | Microscopy

After the ISH was completed, whole-mount embryos and ganglia were placed in 50% glycerol in PBST, either on an agarose plate or a depression slide, or were coverslipped on slides. Ganglia and brain images were collected with Leica Application Suite software (version 4.6.2) with a Leica DFC450 C camera on a Leica M165 FC microscope (Leica Microsystems, Inc., Buffalo Grove, IL). Differential interference contrast images of embryos were captured either with the Leica software and camera described above on a Zeiss Axioskop 2, or with the Zeiss Zen microscope software (ZEN Digital Imaging for Light Microscopy, RRID:SCR_013672) with an Orca Flash 4.0 LT camera (Hamamatsu Photonics, RRID:SCR_017105) on a Zeiss AxioImager M2 microscope. Figures were assembled in Adobe Photoshop (Adobe Photoshop, version 19.1.4; RRID:SCR_014199) and brightness and contrast adjustments were made to some images.

3 | RESULTS

3.1 | Identification of *sema1a* and *plexA* in *G. bimaculatus*

We identified two different *G. bimaculatus sema1a* sequences in silico. A 5′ portion of *sema1a* was identified from a published transcriptome (Zeng et al., 2013), and was confirmed and extended by searching our recent de novo transcriptome assembly, which is based on adult control and deafferented prothoracic ganglia (Fisher et al., 2018). A second, distinct *sema1a* was identified in a draft genome for *G. bimaculatus* (Mito and Noji, personal communication), which we confirmed and extended with a search of our recently assembled transcriptome (Fisher et al., 2018). A majority of both sequences were amplified and confirmed from independent prothoracic ganglion and embryonic samples using PCR and Sanger sequencing. Assuming that these two transcripts led to the translation of two distinct *sema1a* protein products in *G. bimaculatus*, we will refer to the transcript initially identified in the transcriptome as *sema1a.1*, and the transcript initially identified in the draft genome as *sema1a.2*. We recently reported a sequence and domain analysis of the predicted Sema1a.2 protein in *G. bimaculatus* (Fisher et al., 2018), indicating that *D. melanogaster* Sema1a and *G. bimaculatus* Sema1a.2 have the same type, number, and order of predicted protein domains. Searching our transcriptome using the *D. melanogaster* Sema1b protein sequence (AAF57816.1) as a query did not identify a *sema1b* transcript.

When translated, *sema1a.1* was predicted to have a stop codon and a 3′ untranslated region (UTR) while our *sema1a.2* sequence was missing its 3′-most end and did not have a predicted stop codon. The 5′-most ends of each transcript, including the start methionines, have not yet been identified (Figure 1a). Surprisingly, amino acid alignments of the two *G. bimaculatus* Sema1a sequences indicated that they are only about 48% identical to each other (Figure 1a). When these transcripts were used as queries in a BLASTx search, the top matches for both of these putative *sema1a* transcripts were Sema1a proteins found in two species of termites (the top hit for *G. bimaculatus sema1a.1* was *Cryptotermes secundus* Sema1a (XM_023854042.1) and the top hit for *G. bimaculatus sema1a.2* was *Zootermopsis nevadensis* Sema1a (XM_022079449.1).

We also identified several splice variants in *G. bimaculatus sema1a.1* in the region stretching from amino acid 574 to 662 (gray bars in Figure 1a). Further sequencing indicated that this region likely has three different exons that can be alternatively spliced (Figure 1b). All five of these *sema1a.1* splice variants were detected in cDNA derived from the prothoracic ganglia in the CNS of the adult cricket. When using the predicted translation of the longest splice variant (top sequence in Figure 1b) as a query in a conserved domain search, we found that this portion of the protein had some similarity to a ribonuclease E domain, though none of the spliced regions in the shorter variants showed any similarity to conserved domains.

Comparison of the two predicted Sema1a proteins in *G. bimaculatus* to well-characterized Sema1a proteins in *D. melanogaster* and in the grasshopper species *S. americana* revealed 16, well-conserved cysteines in the sema domain (light gray squares in Figure 2a). In addition, a transmembrane domain was predicted in all proteins (as noted in Figure 2a). The putative *G. bimaculatus* Sema1a.1 protein sequence shared more identity with Sema1a in *S. americana* than with Sema1a in *D. melanogaster* (Figure 2b). We did not identify any Sema1a.2-type sequences in GenBank for *S. americana*. Conversely, the predicted *G. bimaculatus* Sema1a.2 protein sequence was more identical to *D. melanogaster* Sema1a (NP_001260265.1) than to Sema1a in *S. Americana* (Figure 2a,b). Searching the data available for *D. melanogaster* in GenBank did not reveal the presence of a Sema1a.1-type sequence. Both of the predicted *G. bimaculatus* proteins share less identity with *D. melanogaster* Sema1b (~35%), Sema2a (~25%), and Sema2b (~25%; Figure 2b).

Several other insects appear to have at least two Sema1a-type proteins as well. Using *G. bimaculatus* Sema1a.1 and Sema1a.2 as queries in tBLASTn searches of non-*Drosophila* arthropod sequences in the GenBank database, we identified multiple cases in both hemimetabolous and holometabolous insects in which at least two Sema1a proteins were predicted for a single species (Table 1). A tanglegram comparing a phylogenetic tree with those proteins that best matched *G. bimaculatus* Sema1a.1 (red) with a tree containing those proteins that best matched *G. bimaculatus* Sema1a.2 (blue), clearly demonstrates the presence of two distinct Sema1a proteins in multiple hemimetabolous and holometabolous insect species (Figure 3). Notably, although the pairs of Sema1a proteins for each species share relatively low levels of amino acid identity (42% identity on average; Figure 3), they are still more similar to Sema1a than they are to any of the other Sema proteins (see Sema2a, 2b, and 1b in black in Figure 3). The expression patterns and functions of these distinct Sema1a proteins are unknown.

We also characterized the main Sema1 receptor, PlexA, in *G. bimaculatus*. We identified a *plexA* transcript predicted to encode a full-length PlexA protein from our assembled transcriptome. Our initial characterization indicates that PlexA in *G. bimaculatus* has the same number, type, and order of protein domains as PlexA (isoform A) in *D. melanogaster* (Fisher et al., 2018). We amplified and confirmed a majority of the predicted sequence from independent prothoracic and embryonic samples using PCR and Sanger sequencing. When using *G. bimaculatus* PlexA as a query, PlexA in the termite *C. secundus* was the top BLAST hit, and distance calculations indicate that *G. bimaculatus* PlexA was 91% identical to PlexA in *C. secundus* and 71% identical to PlexA in *D. melanogaster*. A rooted phylogenetic tree confirms that PlexA in *G. bimaculatus* was more closely related to PlexA in several other insect species than it was to *G. bimaculatus* PlexB (Figure 4). The phylogeny also mirrors the current understanding of the relatedness among insect orders (Misof et al., 2014); PlexA in *G. bimaculatus* was more closely related to PlexA in Isoptera and Hemiptera than to PlexA in Coleoptera and Diptera.

3.2 | Developmental expression of *sema1a.1*, *sema1a.2*, and *plexA* in the cricket embryo

To further characterize these ligands and their receptor, we performed ISH experiments in whole-mount embryos. Images of the

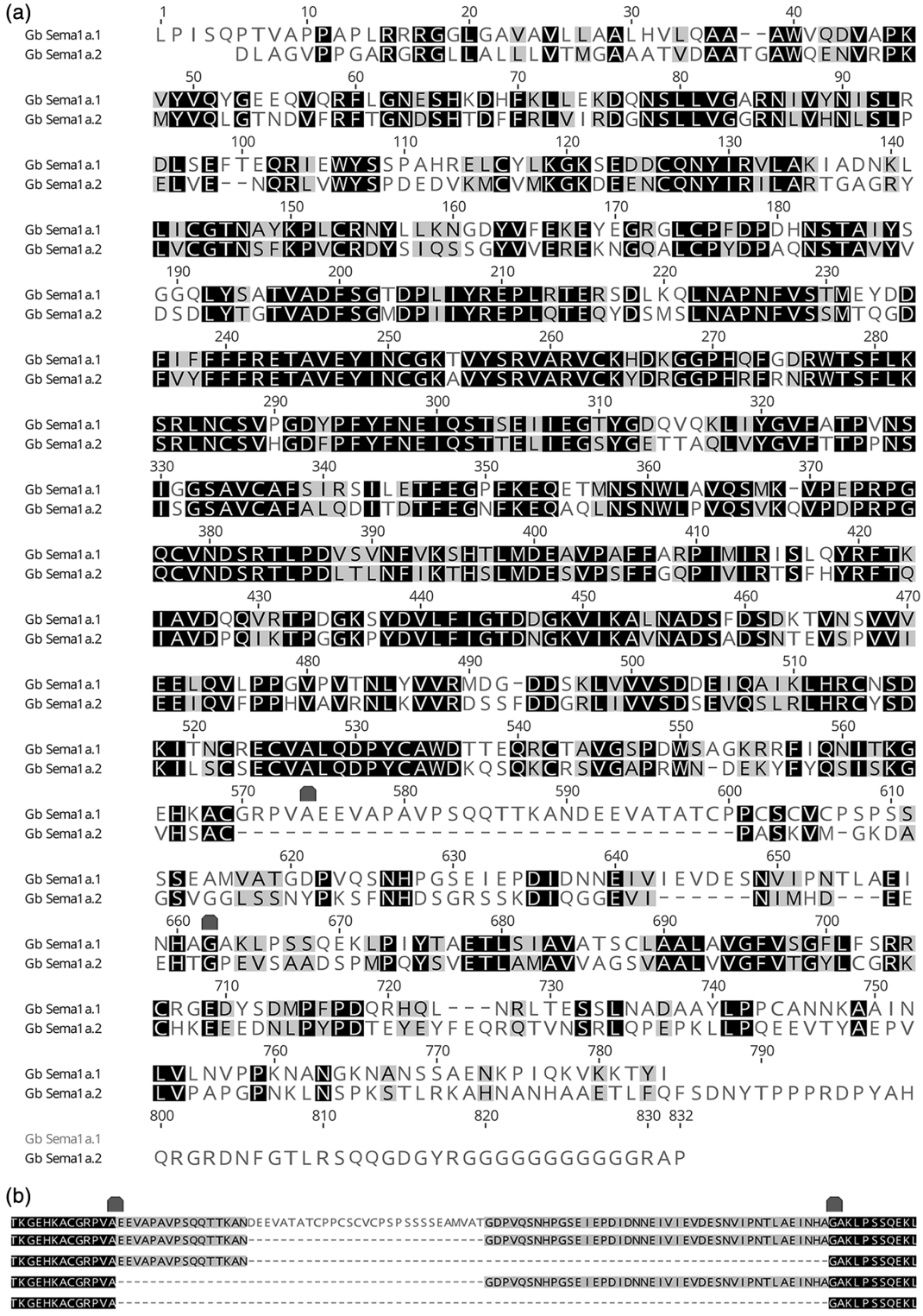

FIGURE 1 Predicted protein sequences for Sema1a.1 and Sema1a.2 in *Gryllus bimaculatus* share 48% identity. (a) Alignment of Sema1a.1 (MK473371) and Sema1a.2 (MF817714.1). Gray bars bookend the region where splice variants have been identified in Sema1a.1. Sema1a.1 has a predicted stop codon, but Sema1a.2 does not. Black shading indicates identical amino acids while gray shading indicates similar amino acids. (b) The sequencing results of five splice variants of Sema1a.1,which were evident after PCR and gel electrophoresis. Gray bars as in (a)

embryonic expression pattern of *sema2a*, which has been thoroughly characterized elsewhere (Maynard et al., 2007), are included for comparison. As noted above, the *G. bimaculatus* Sema1a.1 protein sequence shared a higher level of identity with Sema1a in the grasshopper *S. americana* than in *D. melanogaster*; thus, we predicted that *G. bimaculatus sema1a.1* would be expressed in the developing limb

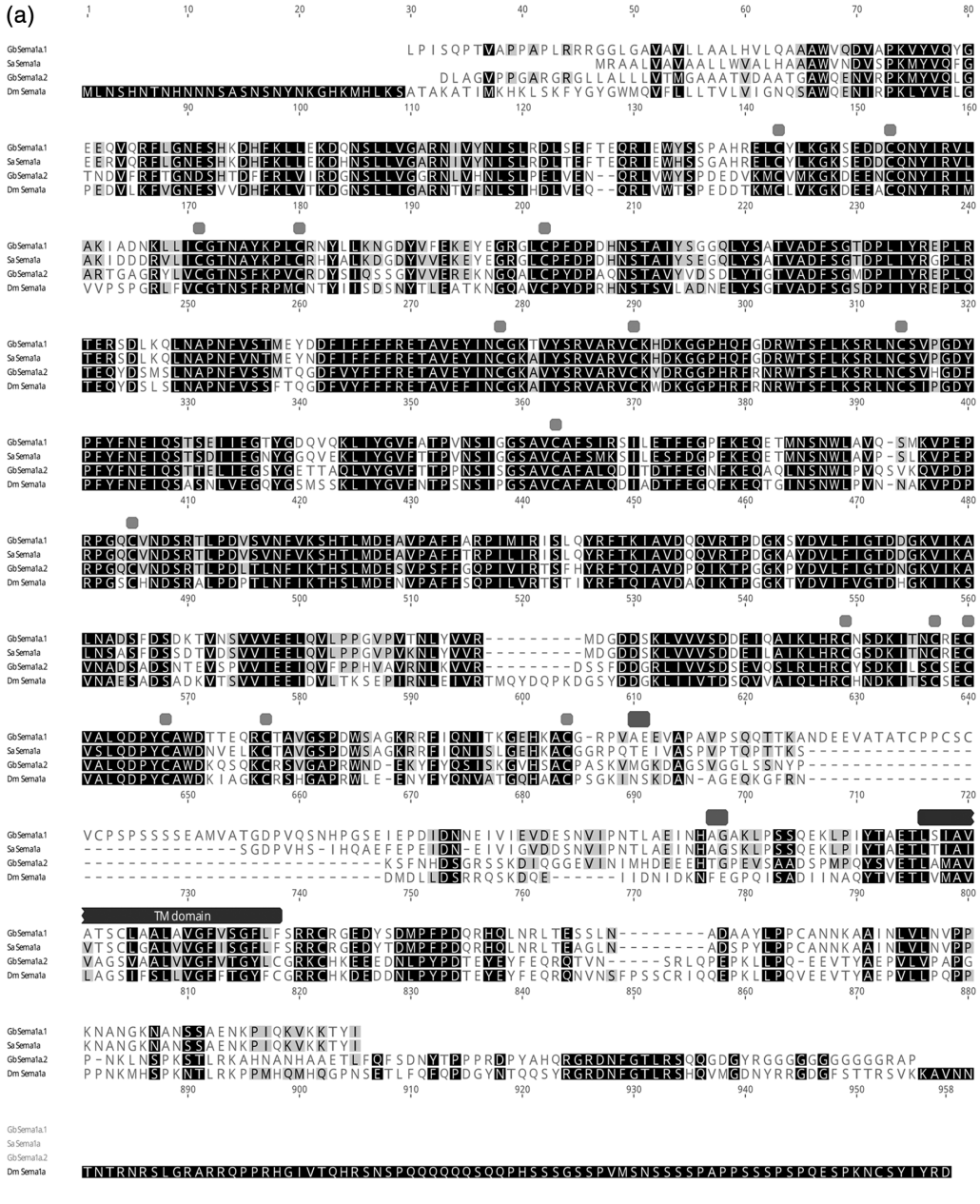

FIGURE 2 Comparisons of Sema1a.1 and Sema1a.2 with Sema1-type proteins in *Drosophila melanogaster* and *Schistocerca americana*. (a) An alignment of *Gryllus bimaculatus* Sema1a.1 and Sema1a.2 with Sema1a from *Schistocerca americana* (AAA29808.1) and *D. melanogaster* (NP_001260265.1). Black shading indicates identical amino acids while gray shading indicates similar amino acids. Cysteines are noted by small gray squares above the sequences. The putative transmembrane domain is also noted by a labeled gray bar above the sequences. Splice variant region bookended by gray rectangles as in Figure 1. (b) A distance matrix showing all the pair-wise percent identity comparisons for Semas in *D. melanogaster* (Dm), *S. americana* (Sa), and *G. bimaculatus* (Gb)

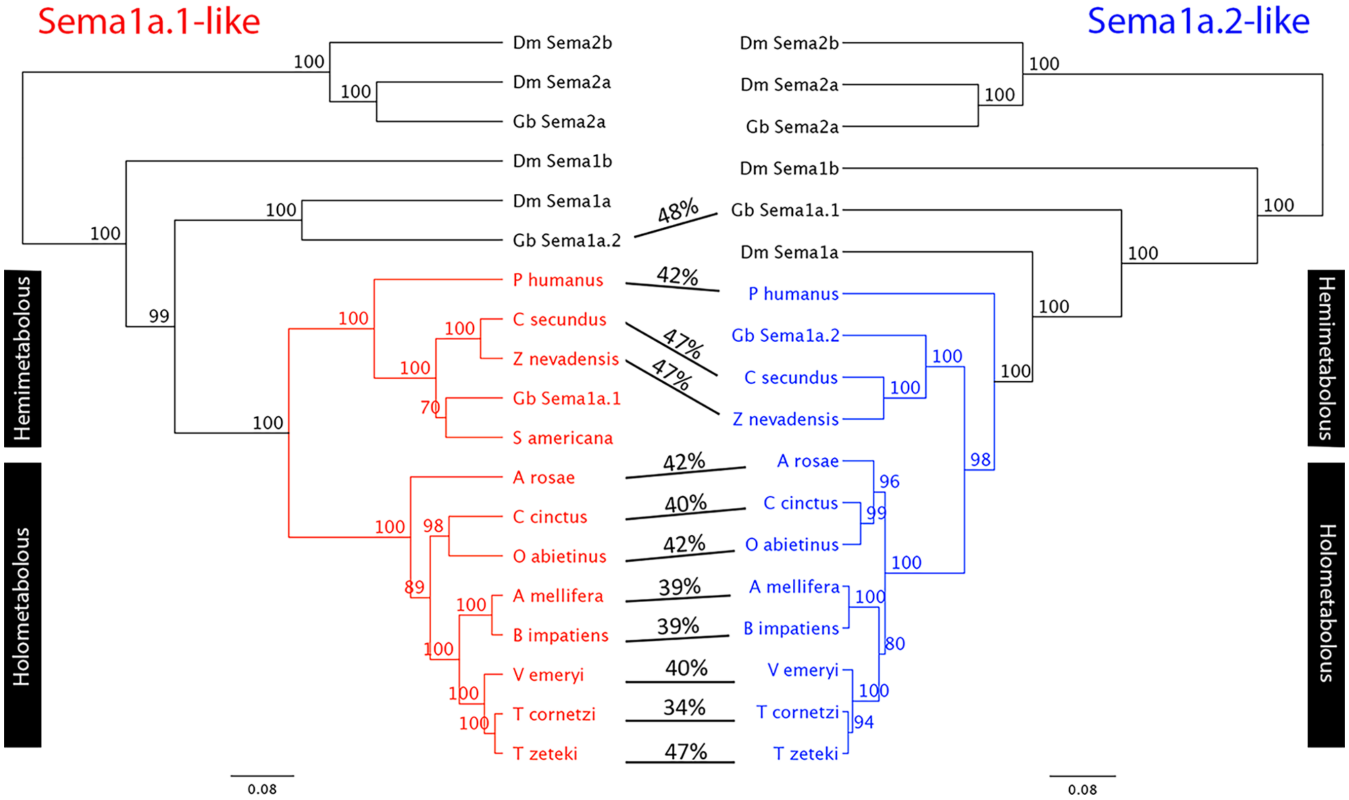

FIGURE 3 A tanglegram showing that multiple hemimetabolous and holometabolous insect species have at least two distinct Sema1a proteins. Two unrooted trees were created using the UPGMA algorithm. Candidates on the left (red) were identified in a tBLASTn search of non-*Drosophila* arthropods using *Gryllus bimaculatus* Sema1a.1 as a query. Candidates on the right (blue) were identified in a tBLASTn search of non-*Drosophila* arthropods using *G. bimaculatus* Sema1a.2 as a query. Groups of hemimetabolous and holometabolous insects are noted. Black lines in the center connect Sema1a-type proteins from each species and % identities are noted. *G. bimaculatus*, *Schistocerca americana*, and *Drosophila melanogaster* Sema proteins were included for reference (in black): *G. bimaculatus* Sema1a.1 (MK473371), Sema1a.2 (MF817714.1), Sema2a (ABK35089.3); *S. americana* Sema1a (AAA29808.1); and *D. melanogaster* Sema1a (NP_001260265.1), Sema1b (NP_611244.1), Sema2a (NP_477507.1), and Sema2b (NP_001261025.1). Genus names are abbreviated. Scale bars at bottom refer to branch lengths and bootstrap values are given at each node. Full species names, common names, and accession numbers of all proteins used in this tree are given in Table 1

buds, similar to observations in *S. americana* embryos (Kolodkin et al., 1992). Indeed, *sema1a.1* expression was most obvious in the developing limb buds (Figure 5a–c) and was not expressed notably in the developing ventral nerve cord (a small amount of nonspecific staining is marked with white asterisks). The expression pattern of *sema2a* in *G. bimaculatus* developing limb buds is included for comparison (Figure 5e–g; Maynard et al., 2007).

The *G. bimaculatus* Sema1a.2 protein sequence shared a higher level of identity with Sema1a in *D. melanogaster* than it did with Sema1a in *S. Americana*; thus, we predicted that *G. bimaculatus sema1a.2* would be expressed in the CNS, similar to observations in *D. melanogaster* embryos (Kolodkin et al., 1993). In fact, *sema1a.2* expression was evident in the developing ventral nerve cord but was absent in the developing limb buds in *G. bimaculatus* (Figure 5i–k). *sema1a.2* expression was not evident in Stage 6 embryos (19% development; n = 2; data not shown) and only light staining was seen near the midline in early Stage 7 embryos (25% development; n = 6; data not shown). Late Stage 7 (29–32% development) and Stage 8 embryos (33–41% development), however, showed robust CNS staining (n = 7; Figure 5i–k), as did Stage 9 embryos (42% development; n = 4, data not shown). The expression in the ventral nerve cord can be seen in distinct patches on either side of the midline (Figure 5k). There was very little *sema1a.2* expression outside the developing CNS (Figure 5i). All three semaphorins, *sema1a.1*, *sema1a.2*, and *sema2a*, were expressed in embryonic cricket brains (Figure 5d,h,l). The expression pattern of each transcript appeared widespread across the brain, though symmetrical regions of stronger *sema2a* and *sema1a.2* expression were evident (arrows in Figure 5h,l).

The expression of *plexA* in embryos overlapped with the general regions of expression for both *sema1a.1* in the limb buds and *sema1a.2* in the CNS (n = 15). *plexA* expression was clearly visible on either side of the midline in the ventral nerve cord (Figure 5m,n). When comparing the *sema1a.2* and *plexA* expression patterns, it appeared that *plexA* was expressed most strongly on the lateral edge of the ventral nerve cord, and less so in the medial region (Figure 5n). *plexA* was also expressed in the developing limb buds in distinct patterns (Figure 5n,o). Global *plexA* expression was also evident in the embryonic brain (Figure 5p).

To understand the expression patterns of *sema1a.1* and *plexA* in developing limb buds more fully, we compared expression of each

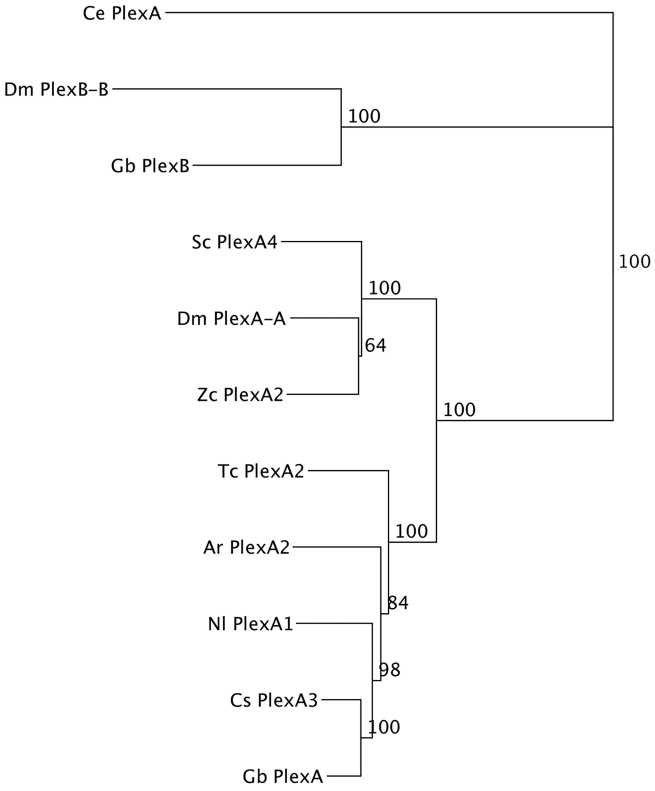

FIGURE 4 Phylogenetic tree showing the relatedness of PlexA in *Gryllus bimaculatus* with PlexA in other invertebrates. This tree was rooted using PlexA from *Caenorhabditis elegans* (BAB85224) and was created using the Neighbor Joining algorithm. It includes PlexA proteins from the stable fly *Stomoxys calcitrans* (XP_013102512), the fruit fly *D. melanogaster* (NP_524637.2), the melon fly *Zeugodacus cucurbitae* (XP_011193231), the red flour beetle *Tribolium castaneum* (XP_008199067), the sawfly *Athalia rosae* (XP_020708811), the planthopper *Nilaparvata lugens* (XP_022188299), the termite *Cryptotermes secundus* (XP_023712882), and the cricket *G. bimaculatus* (MK473372), as well as PlexB from *D. melanogaster* (NP_001245400) and *G. bimaculatus*. Species names are abbreviations of the genus and species, bootstrap values are given at each node

transcript in prothoracic legs across a range of developmental stages. *sema1a.1* expression was evident in the limb buds of most embryos staged 7 (23% development) through Stage 9.5 (49% development; Figure 6), though expression was more often evident as spots instead of bands prior to Stage 8.5, such as at Stage 7 (Figure 6a arrowheads) and Stage 8 (37% development; arrowheads in Figure 6c). For example, in Stage 7 embryos, two of eight embryos had *sema1a.1* expressed exclusively in spots, one showed spots with some banding, and one had only bands. Half the embryos from this age range lacked clear staining (data not shown). Most of the spots evident in Stages 7 and 8 embryos consisted of clusters of several cell bodies (arrowheads in Figure 6a,c, and inset in Figure 6c). By Stage 8.5, expression for *sema1a.1* appeared to transition to a banding pattern in most limbs examined (Figure 6e; n = 9/14), though three embryos at this stage had only spots and two embryos showed no obvious staining pattern (data not shown). Finally, in Stages 9 and 9.5 embryos (42–49% development; Figure 6g,i) the majority of embryos possessed bands of *sema1a.1* expression (n = 22/26); two embryos had only spots and two embryos lacked obvious staining (data not shown). An example of *sema1a.1* expression in both spots (arrowheads) and bands (arrows) in the limb buds of a Stage 9.5 embryo (42% development) can be seen in Figures 5c and 6i.

plexA was also expressed in a combination of bands and stripes (Figures 5o and 6). Spots of *plexA* were obvious in limb buds in embryos through Stage 8 (33–41% development; n = 8). They were particularly noticeable on the dorsal edge near the tibiofemoral joint (arrowheads Figures 5o and 6d,f), while *plexA* was expressed in a broader pattern at the distal tip of the tarsus (arrows, Figures 5o and 6d,f). Spots of *plexA* expression at these early stages consisted of several cell bodies (Figure 6d,f, arrowheads, and inset in Figure 6f). By Stage 9 (42% development), *plexA* appeared in diffuse stripes (Figure 6h; n = 3), while stripes were more distinct in limb buds of Stage 9.5 embryos (49% development; arrows Figure 6j; n = 4).

TABLE 1 At least two Sema1a-type proteins were identified in a number of insect species

Species	Common name	tBLASTn hits for *Gb* Sema1a.1 query	tBLASTn hits for *Gb* Sema1a.2 query
Apis mellifera	Honey bee	XP_016766207.1	XP_394163.3
Athalia rosae	Turnip sawfly	XP_020710981.1	XP_012260933.1
B. impatiens	Bumble bee	XP_024223929.1	XP_003492080.1
Cephus cinctus	Wheat stem sawfly	XP_015594723.1	XP_015603711.1
Cryptotermes secundus	Drywood termite*	XP_023709809.1	XP_023725893.1
Orussus abietinus	Parasitic wood wasp	XP_012285376.1	XP_012277064.1
Pediculous humanus	Human louse*	XP_002423485.1	XP_002424651.1
Trachymyrmex cornetzi	Fungus growing ant	XP_018373672.1	XP_018376122.1
Trachymyrmex zeteki	Fungus growing ant	XP_018315790.1	XP_018317585.1
Vollenhovia emeryi	Ant (no common name)	XP_011864803.1	XP_011869172.1
Zootermopsis nevadensis	Dampwood termite*	XP_021930953.1	XP_021935130.1

Accession numbers for tBLASTn hits from 11 different insect species in response to using either Sema1a.1 (red) or Sema1a.2 (blue) from *G. bimaculatus* as a query. (See Figure 3 for related phylogenetic tree). The common names marked with an asterisk are hemimetabolous insects, as is *G. bimaculatus*. All other insects are holometabolous.*

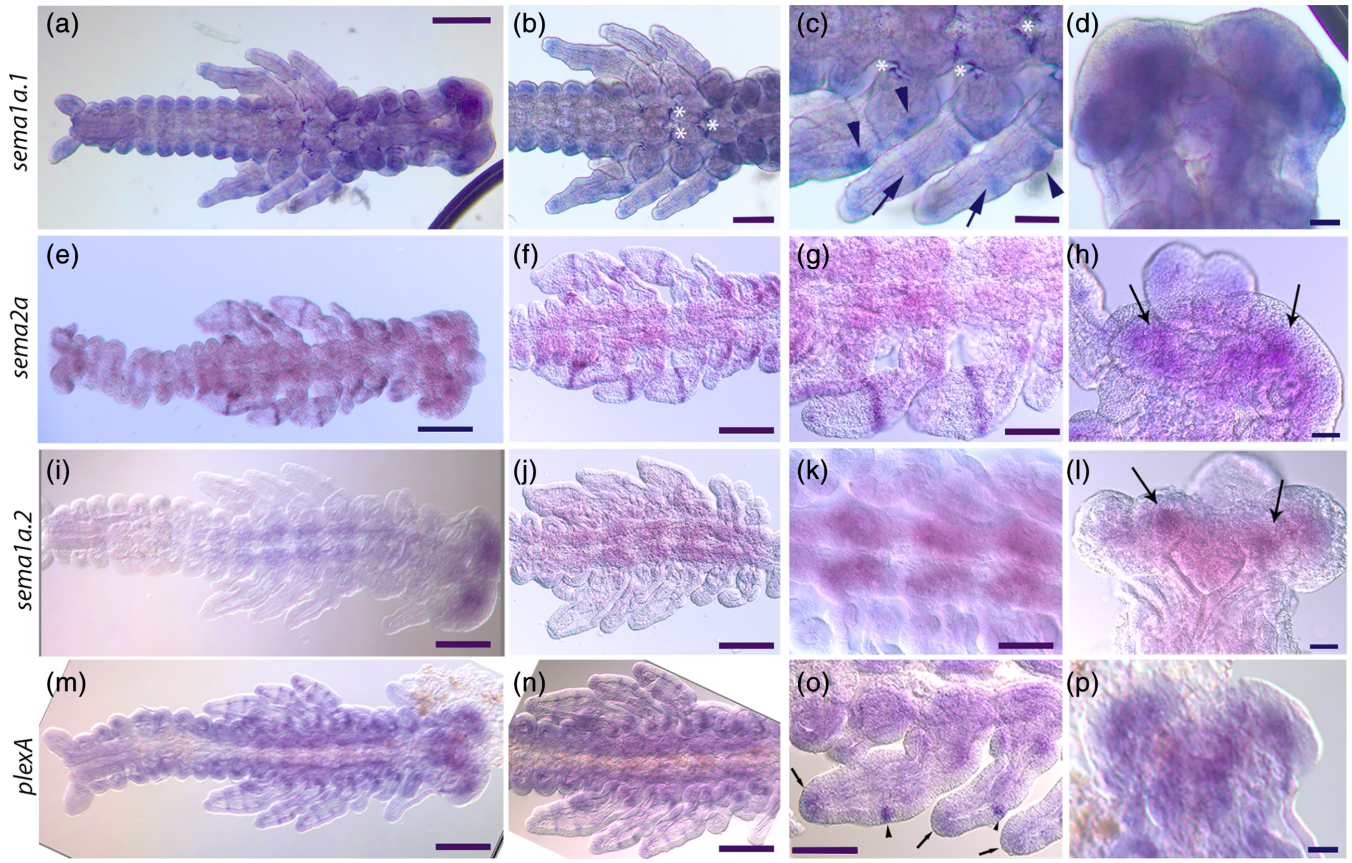

FIGURE 5 *sema1a.1*, *sema1a.2*, and *plexA* are expressed in the developing embryo. (a) *sema1a.1* was expressed in the developing limb buds and brain. (b) Distinct *sema1a.1* staining was seen in the limb buds, but little expression was evident near the midline of the CNS. Nonspecific staining near the midline is noted with white asterisks. (c) *sema1a.1* expression in the limb buds of a Stage 9.5 embryo was evident as mix of stripes (arrows) and distinct spots (arrowheads). Nonspecific staining marked with white asterisk. (d) *sema1a.1* was expressed across the developing brain. (e–g) *sema2a* was expressed in stripes in the developing limb buds. (h) *sema2a* was expressed in the developing brain, and bilateral spots of more intense staining were evident (arrows). (i,j) *sema1a.2* was expressed in the developing ventral nerve cord but absent from the limb buds. (k) *sema1a.2* expression can be seen on either side of the midline in the developing ventral nerve cord. (l) *sema1a.2* was expressed in the developing brain, and bilateral spots of more intense stating were evident (arrows). (m) *plexA* was expressed both in the limb buds and in the ventral nerve cord of a Stage 8 embryo. (n) The pattern of *plexA* expression in the ventral nerve cord was more lateral than that of *sema1a.2*. (o) In Stage 8.5 embryo, *plexA* expression was limited to distinct spots along the epithelial margin near the joint between the tibia and femur (arrowheads) and at the distal tip of the tarsus (arrows). (p) *plexA* was also expressed in the developing embryo brain. Scale bars in the first column of images (a,e,i,m) = 200 μm. Scale bars in the second column of images (b,f,j,n) = 100 μm. Scale bars in the third column of images (c,g,k, o) = 50 μm. Scale bars in the last column (d,h,l,p) = 50 μm

3.3 | Expression of *sema1a.1*, *sema1.2*, and *plexA* in the adult CNS

We used ISH in whole-mount adult *G. bimaculatus* brains to compare and contrast the expression of *sema1a.1*, *sema1a.2*, and *plexA*, with that of *sema2a*, which we have previously described (Maynard et al., 2007). While *sema2a* expression was obvious in the MBs of the adult brain (Figure 7d–f; Maynard et al., 2007), *sema1a.1* was only weakly expressed (n = 4; Figure 7a–c). The most anterior region, including the MBs, did have some light expression (Figure 7b), though no distinct ring at the very tip of the MB was seen (Figure 7d). A dark ring of stain in this area was obvious for *sema2a* (arrow, Figure 7f). In contrast with *sema1a.1*, *sema1a.2* was expressed fairly robustly in the MBs and more diffusely in a region just posterior to the MBs (n = 4; Figure 7g, h). Expression was slightly stronger at the edge of the MB, seen as a ring at the edge of the Kenyon cell region (arrows in Figure 7h) as well as in a faint ring around the neurogenic tip (bottom arrow in Figure 7i). A high-power image of the Kenyon cells shows individual cells approximately 6 μm in diameter with *sema1a.2* expression most evident at the margins of the cells (Figure 7j). *sema1a.2* expression appeared qualitatively weaker than that of *sema2a*, though no quantification was performed.

We also characterized the expression pattern of *plexA* in the adult brain. *plexA* expression in the brain was not as robust as that of *sema2a and sema1a.2* (n = 4; Figure 7k,l), but appeared slightly stronger than that seen for *sema1a.1*. In addition, no distinct *plexA* expression was apparent at the tip of the mushroom body near the proliferative zone (Figure 7k), though a region of darker stain at the base of the MB region was seen (arrows in Figure 7k,l). The use of sense probes consistently showed a complete lack of staining

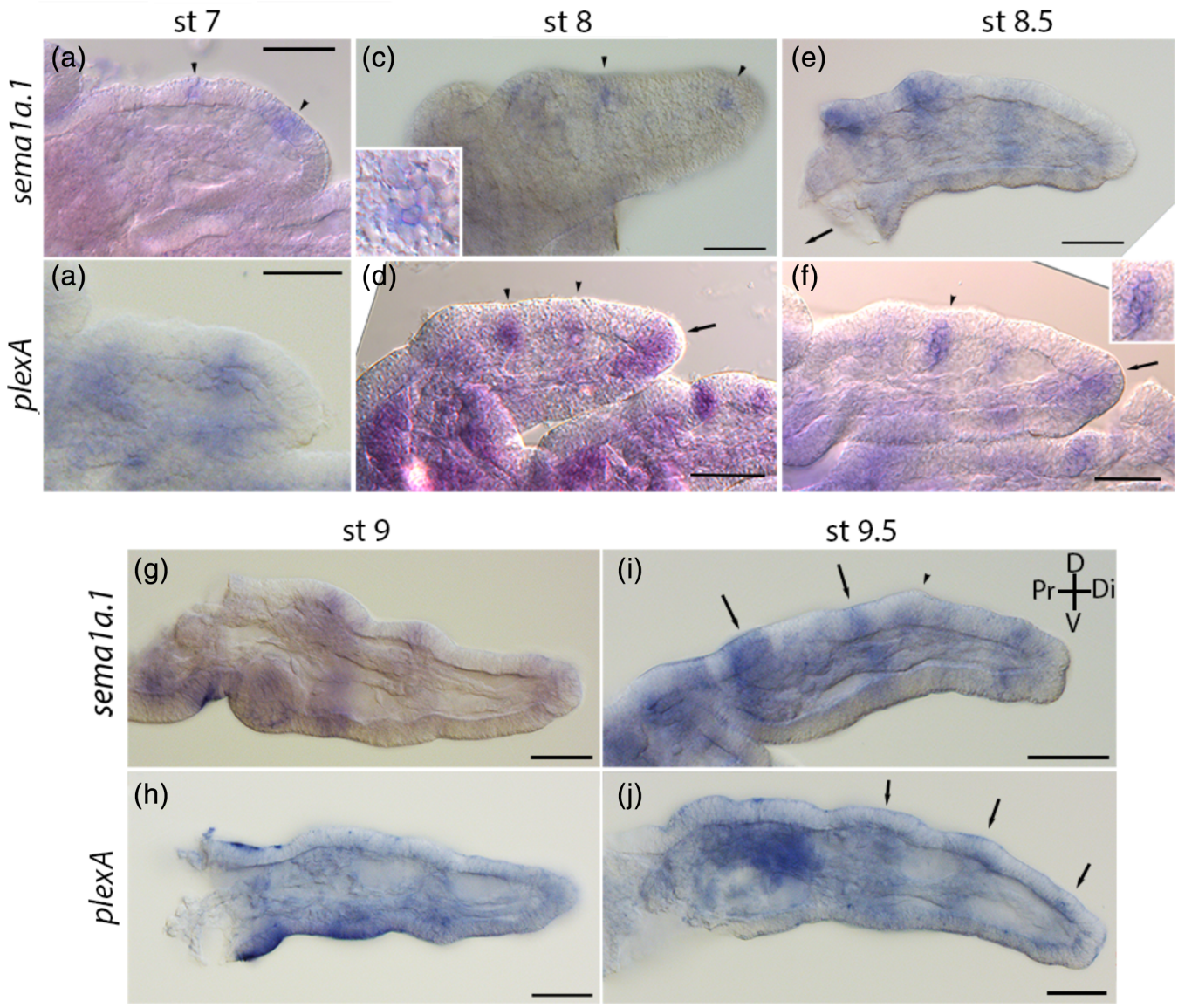

FIGURE 6 Comparison of *sema1a.1* and *plexA* expression in developing prothoracic limbs at several different developmental stages. (a) Two distinct spots of *sema1a.1* expression were visible in Stage 7 limb buds (arrowheads). (b) *plexA* expression was also visible at this stage but the pattern was broader. (c) Two distinct spots of *sema1a.1* expression were found in the prothoracic limb of most Stage 8 embryos (arrowheads). The most distal spot is shown at higher power in the inset on the left. Spots consist of a small number of cells. (d) Three regions of *plexA* expression were evident in prothoracic limbs of most Stage 8 embryos as a combination of spots (arrowheads) and broader regions (arrow). (e) In Stage 8.5 embryo prothoracic limbs, *sema1a.1* expression was evident as broad stripes. (f) In Stage 8.5 embryo prothoracic limbs, *plexA* expression was a mix of spots and broader regions of expression. The arrowhead highlights a spot consisting of several cell bodies. The region noted by the arrowhead is magnified in the upper right inset. (g) *sema1a.1* expression was evident in Stage 9 prothoracic limbs as a mix of full and partial stripes. (h) At Stage 9, *plexA* expression was evident as broad stripes in prothoracic limb. (i) At Stage 9.5, *sema1a.1* expression was a mix of stripes (arrows) and more distinct spots (arrowhead). (j) *plexA* expression in Stage 9.5 prothoracic limbs was mostly in broad stripes (arrows). Dorsal (D), ventral (V), proximal (Pr), and distal (Di) orientations are noted in (i) and apply to all developing limbs. Scale bars in all images = 50 μm

(*sema1a.2* sense example in Figure 7m) indicating that the expression patterns and levels observed for *sema1a.1* and *plexA*, though non-distinct and fairly weak, were likely specific.

In adult thoracic ganglia, *sema1a.1*, *sema1a.2*, and *sema2a* were all expressed (Figure 8a–i), although *sema1a.1* expression was quite weak (Figure 8a–c). Faint *sema1a.1* expression can be seen in the posterior and anterior regions of the prothoracic (Figure 8a; n = 4) and mesothoracic ganglia (Figure 8b; n = 4), though staining was not clearly evident in the metathoracic ganglia examined (Figure 8c; n = 3). *sema2a* was expressed more strongly and was evident in all three ganglia (Figure 8d–f). The expression of *sema1a.2*, however, was quite robust in all three thoracic ganglia (Figure 8g–i; n = 8), with most cells in the anterior and posterior regions expressing this transcript. Given that PlexA is the main receptor for the Class I Semas,

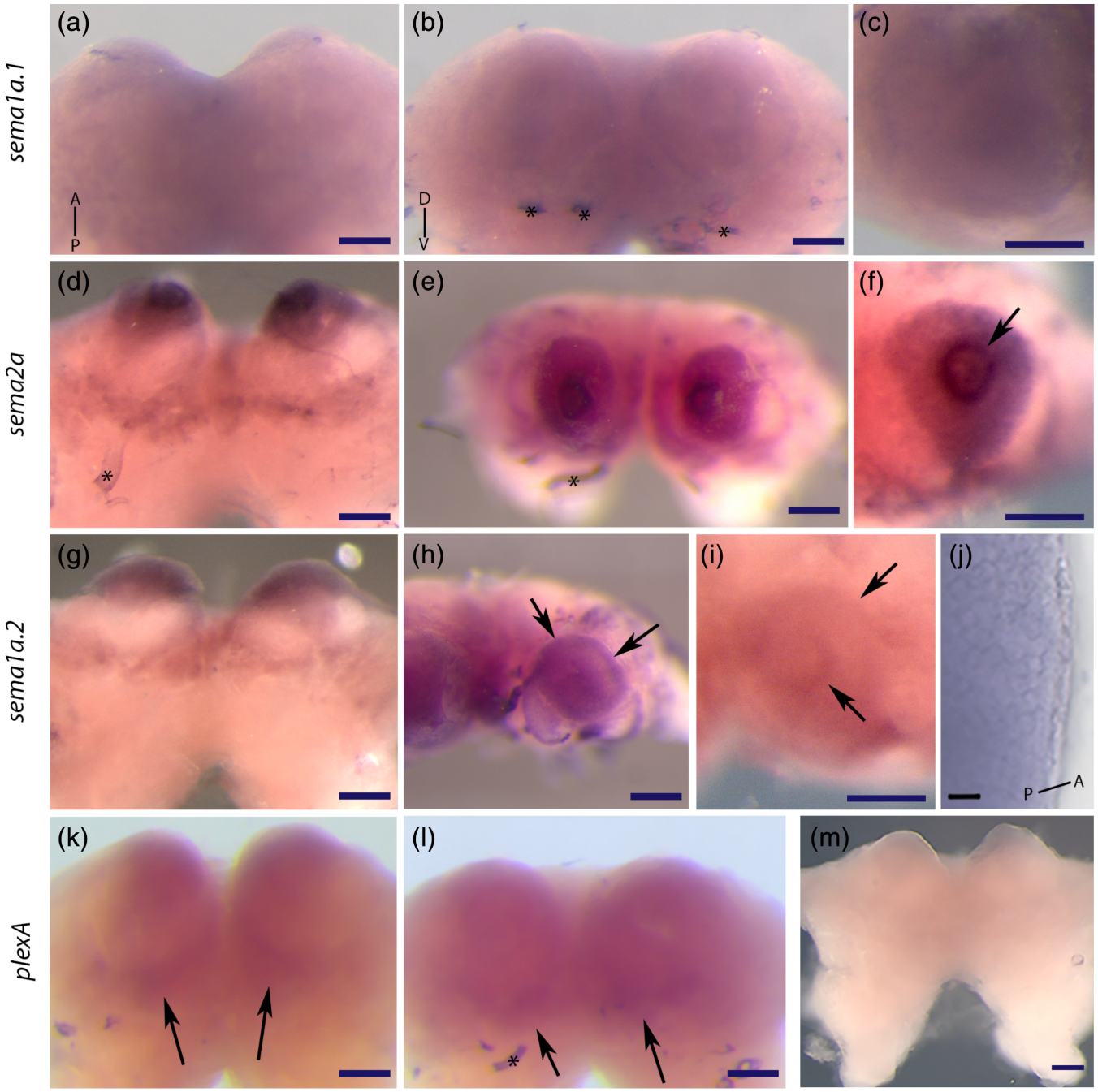

FIGURE 7 *sema1a.2* and *sema2a* are clearly expressed in the mushroom bodies of the adult brain. (a) *sema1a.1* expression was weak throughout the brain. (b) Some limited *sema1a.1* stain was evident in the most anterior end of the brain. (c) *sema1a.1* staining in the MB region was detectable, though no distinctive ring around the neurogenic tip was evident. (d) *sema2a* was expressed strongly in the anterior region of the brain, especially in the MBs and in a region below the MBs. (e,f) The strongest *sema2a* expression was seen in a ring at the tip of each mushroom body (arrow in f). (g) *sema1a.2* was expressed most strongly in the anterior region of the brain, including the MBs. (h) *sema1a.2* expression in the MBs was strongest in two rings, one at the edge of the Kenyon cell region (arrows), (i) and a second ring was faintly visible at the neurogenic tip of the MB (arrows highlight inner and outer ring). (j) A high power image of the Kenyon cell region shows staining at the margins of the cells. (k) *plexA* was weakly expressed in the most anterior region of the brain, including in the MB, and diffusely more posterior to the MB. (l) No distinct ring of expression was seen at the tip of the MB. (m) Use of a sense *sema1a.2* probe showed no obvious staining. Asterisks indicate trachea, which tend to trap probe. Scale bar in (j) = 10 μm all others = 200 μm. Anterior and posterior, in relation to the neuraxis, are indicated in the bottom left of the image in (a), which also applies to the left column of images and to the image in (l) and (m). The anterior–posterior neuraxis is also noted in (j). The remaining images are viewed from the anterior end of the brain with ventral and dorsal indicated as in (b)

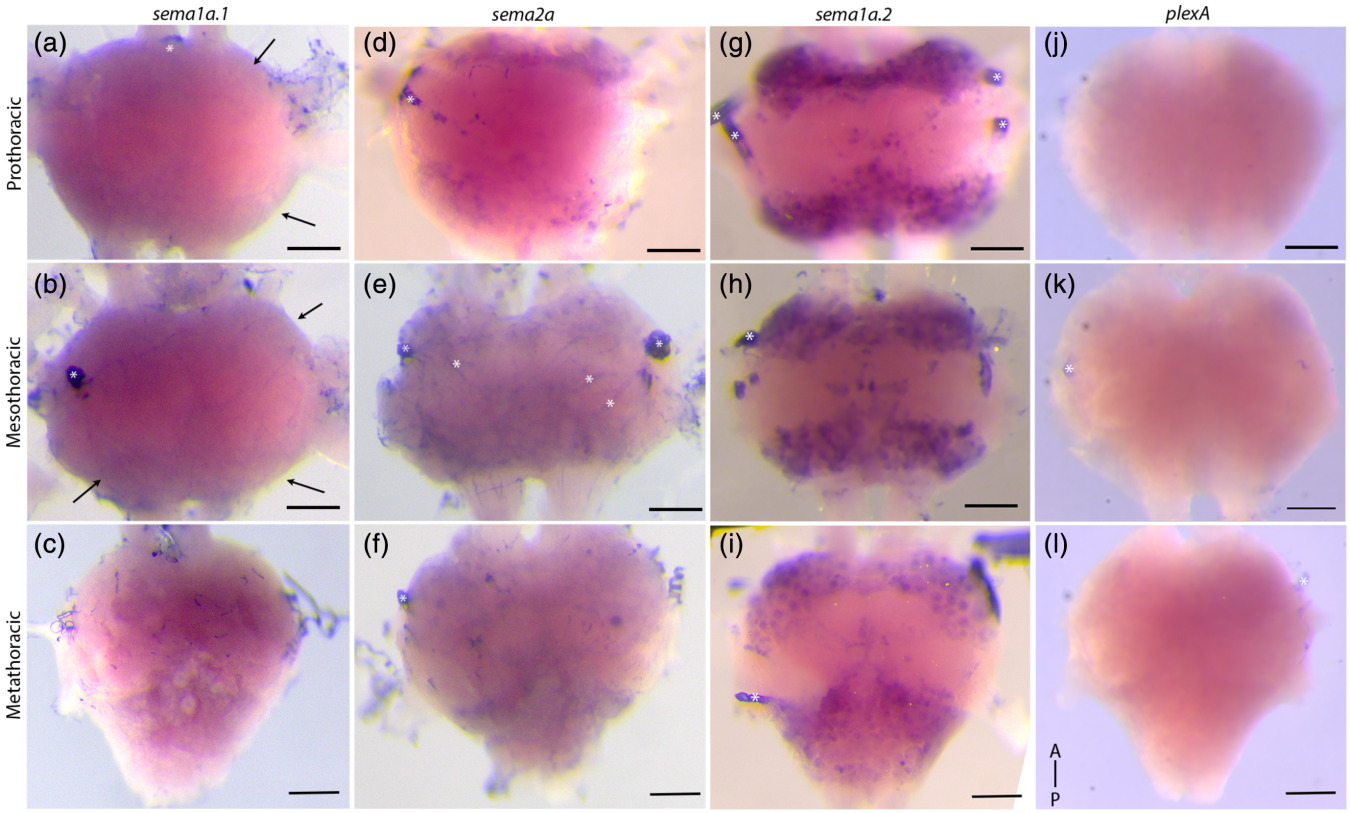

FIGURE 8 Expression patterns for *sema1a.1*, *sema1a.2*, and *sema2a* were evident in all three adult thoracic ganglia, but *plexA* was absent. *sema1a.1* expression in adult (a) prothoracic (b) mesothoracic ganglia was present but weak. Arrows in (a) and (b) highlight regions of expression. (c) *sema1a.1* expression in adult metathoracic ganglia was not detectable. *sema2a* expression was evident in the posterior and anterior regions of the (d) prothoracic, (e) mesothoracic, and (f) metathoracic ganglia. *sema1a.2* was expressed robustly in most cell bodies in the anterior and posterior regions of the (g) prothoracic, (h) mesothoracic, and (i) metathoracic ganglia. (j–l) *plexA* mRNA expression was not detectable in any of the adult thoracic ganglia. White asterisks indicate nonspecific staining of trachea, which tend to trap probe. The anterior and posterior axis for all images is noted in (l). All scale bars = 200 μm

and since we observed expression of both *sema1a.1* and *sema1a.2* in this ganglion, we were surprised by the lack of obvious *plexA* expression in any of the adult thoracic ganglia (Figure 8j–l; $n = 5$).

4 | DISCUSSION

We have characterized two distinct *sema1a* transcripts, *sema1a.1* and *sema1a.2*, in the cricket, *G. bimaculatus*, along with a *plexA* transcript presumably encoding their receptor. The expression of these two *sema1a*-type transcripts map predictably onto patterns previously described in developing *D. melanogaster* (Kolodkin et al., 1993) and the grasshopper *S. americana* (Kolodkin et al., 1992). In the adult brain and thoracic ganglia, *sema1a.2* was expressed more strongly than either *sema1a.1* or *plexA*. Although *plexA* was detectable in the MBs of the adult brain, it was difficult to detect in any cells expressing *plexA* in adult thoracic ganglia, raising questions about Sema signaling mechanisms in the adult CNS.

4.1 | Phylogenetic analysis of Sema1a-type proteins

Available evidence supports our assertion that we have identified two *sema1a*-type transcripts instead of *sema1b* or *sema2a* homologs. The percent identity among the predicted *G. bimaculatus* Sema proteins indicates that these two Sema1a-type proteins share more identity with Sema1a candidates than with non-Sema1a candidates. In addition, a specific search for *sema1b* in our transcriptome did not identify any candidate sequences (Fisher et al., 2018). There are many technical reasons that *sema1b* may not be represented in our transcriptome, but we have reason to suspect that it is truly absent. ISH experiments in *D. melanogaster* have shown that *sema1b* is not expressed in the PNS or CNS of the developing embryo, but is instead present in early oocytes and at the embryonic midline during gastrulation (Khare, Fascetti, DaRocha, Chiquet-Ehrismann, & Baumgartner, 2006). This distinction could explain the absence of *sema1b* in our transcriptome, which is derived from adult CNS tissue (Fisher et al., 2018). Moreover, a recent search of the GenBank database indicates that Sema1b may be limited to Diptera, indicating that this protein might be a result of a fairly recent gene duplication. In contrast, the two *sema1a*-type transcripts described here are robustly expressed in both the periphery and CNS in developing *G. bimaculatus*.

Multiple species across much of Insecta possess predicted orthologs of both Sema1a.1 and Sema1a.2 as well. We found two Sema1a-type proteins in a range of hemimetabolous (lice, termites,

and crickets) and holometabolous (wasps, bees, sawflies, and ants) insects. A recent search of the GenBank database indicates that *S. americana* has only one Sema1-type protein (which is most similar to Sema1a.1). Similarly, in another grasshopper species, *S. gregaria* Sema2a is the sole identified Sema variant. It seems unlikely, however, that these species exclusively possess single Sema proteins. Rather, the absence of other Sema proteins is likely due to the incompleteness of available genomic and transcriptomic data. Further sequencing efforts in other insect species along with characterization of *sema1a*-type transcripts in these species would help confirm their presence in insects outside of *G. bimaculatus*.

For convenience in identifying and distinguishing these two transcripts, we have here adopted the "1a.1" and "1a.2" naming convention; however, further phylogenetic and functional analyses would need to be completed in other insects in order to evaluate the most appropriate names for these transcripts. For example, should one of these predicted proteins be named Sema1c? Given that Sema1a.1 and Sema1a.2 in *G. bimaculatus* share more protein sequence identity (48%) than do Sema1a and Sema1b in *D. melanogaster* (32%), it is not clear if the use of Sema1c is warranted.

Only two known Plexin proteins, PlexA and PlexB, have been found in invertebrates. A single *plexA* transcript was predicted from our *G. bimaculatus* transcriptome. This putative PlexA protein appeared to be fairly well-conserved, sharing reasonably high levels of identity with PlexA in *C. secundus* (termite) and *D. melanogaster*. As previously characterized, *G. bimaculatus* PlexA shares the same domain type, number and order as compared to *D. melanogaster* PlexA (Fisher et al., 2018), indicating that this protein's structure, and presumably its functions, are well-conserved.

4.2 | Sema1a.1, Sema1a.2, and PlexA in development

Much of what we know about the function of Sema1a-type and PlexA proteins in insect CNS development has come from work in *D. melanogaster*; however, recent work in mosquito, mainly in *A. aegypti*, has deepened our understanding of the well-conserved functions of these proteins while also highlighting their distinct functional roles in different species. For example, Sema1a has been shown to be critical for the development of the ventral nerve cord in both *D. melanogaster* (Winberg et al., 1998; Yu et al., 1998) and *A. aegypti* (Haugen et al., 2011). In *A. aegypti*, Sema1a knockdown results in thinning of longitudinal tracts, although this thinning is not detected in similar knockout experiments in *D. melanogaster*. In *D. melanogaster*, PlexA and Sema1a are clearly required for the formation of the most lateral longitudinal fascicle (Wu et al., 2011), although they are not necessary for such formation in *A. aegypti* (Haugen et al., 2011). Sema1a is critical to the proper development of the visual system (Cafferty, Yu, Long, & Rao, 2006; Mysore et al., 2014) and the olfactory system (Komiyama, Sweeney, Schuldiner, Garcia, & Luo, 2007; Mysore, Flannery, Tomchaney, Severson, & Duman-Scheel, 2013), though important differences in the exact nature and timing of Sema1a function in these systems are evident (Mysore et al., 2013, 2014). Further work in the *G. bimaculatus* will deepen our understanding of the conserved functions of Sema signaling in CNS insect development.

In the grasshopper, the Sema1a protein (then known as FasIV) is expressed in a subset of axon pathways in the developing CNS (Kolodkin et al., 1992). Although we did not visualize Sema1a.1 protein distribution in the cricket CNS, the presence of *sema1a.1* transcript in small groups of cell bodies in the limb buds might mean that Sema1a.1 protein would be expressed in axons that grow into the CNS. Although *sema1a.2* was expressed robustly in the CNS, the lack of *plexA* right at the midline might limit Sema1a.2 signaling via PlexA to the lateral edge of the developing CNS. Knockdown experiments or mutational analyses in *G. bimaculatus* would reveal whether the function of Sema signaling in ventral nerve cord development is similar to that seen in Diptera. Alternatively, if these experiments revealed that Sema and Plex gene function diverged in Orthoptera, it would provide clues about the origins of gene function diversity in this family of guidance molecules.

Portions of the developing PNS are also reliant on Sema proteins for proper development. Experiments in grasshopper show that the guidance of tibial pioneer axons relies on the function of both Sema2a and Sema1a for accuracy (Isbister et al., 1999; Kolodkin et al., 1992; Wong et al., 1999). In *G. bimaculatus*, *sema1a.1* expression appeared first in distinct spots in Stages 7 and 8 embryos (23–33% development), and then appeared in bands by Stage 8.5 (37%). About half the Stage 7 embryos we examined did not have any staining in the limbs, indicating some variability in when these spots began to appear during development. It is informative to compare the mRNA expression timing and patterns in *G. bimaculatus*, as assessed with ISH, with the protein timing and patterns in grasshoppers, as assessed by immunohistochemistry (Isbister et al., 1999; Kolodkin et al., 1992). In grasshopper, stripes of Sema1a protein are obvious at 34.5% development (Kolodkin et al., 1992) and appear as early as 32% development (Isbister et al., 1999). This is soon after the newborn tibial pioneer neurons, born at the distal tibial tip, appear at 30% development (Bate, 1976; Keshishian & Bentley, 1983). Stripes of *sema1a.1* in *G. bimaculatus* appear a bit later (37%) than that described in the grasshopper (32%). The exact time of the appearance of tibial pioneer neurons has not been described in *G. bimaculatus*, but in the cricket *T. commodus*, they are present by 30% development and can be destroyed with heat shock at the 27% stage (Klose, 1996). The distinct spots we see prior to Stage 8.5 have not been described in the grasshopper, though no in situ data for *sema1a.1* exists in that species. Comparing the mRNA and protein expression of Sema1a.1 in developing limb buds in *G. bimaculatus* would help clarify similarities and differences in these two species.

Given the role of Sema1a in the guidance of neurons in the grasshopper limb bud (Isbister et al., 1999; Wong et al., 1999), we had expected *plexA* expression in the limb buds to be limited to the soma of developing neurons. The fairly distinct spots of *plexA* expression prior to Stage 8.5 (37% development; Figure 6b,d) might be made up of a small number of growing neurons that need to express PlexA in order to respond to Sema1a.1 cues. All the spots we have observed, however, are made up of more than two cell bodies (Figure 6d,f). The spots

on the dorsal edge of the limb could be cells developing into a sensory organ, such as the subgenual organ, though the early stages of development of these organs have not been fully described in *G. bimaculatus* (but see Klose, 1996). The subsequent expression of *plexA* in stripes, however, was surprising. Perhaps stripes of *sema1a.1* and *plexA* are required for further development of the leg or maintenance of axonal pathways. PlexA is not known to work in a Sema-independent manner, though a genome-wide screen in *D. melanogaster*, recently identified the existence of a circular *plexA* RNA (Westholm et al., 2014), raising the possibility that *plexA* RNA itself could have novel functions.

4.3 | Sema1a signaling in the adult

The expression of many guidance molecules tends to decrease after development, though expression appears to be maintained in select areas. In particular, areas with high levels of plasticity, such as the hippocampus in mammals (Giger et al., 1998; Pascual, Pozas, & Soriano, 2005; Sahay et al., 2005) or the MBs in invertebrates (Eickhoff & Bicker, 2012; Maynard et al., 2007), continue to express developmental guidance molecules, including the Sema proteins, into adulthood. Experiments in mammals indicate that these developmental guidance proteins are important regulators of adult neuroplasticity, pruning axons, eliminating synapses, and reducing spine density and size in order to constrain plasticity in more mature circuits (Boggio et al., 2019; Lee et al., 2012; Riccomagno & Kolodkin, 2015; Tran et al., 2009).

We have previously documented the expression of *sema2a* in the adult brain of *G. bimaculatus* (Maynard et al., 2007), and here we show that *sema1a.1*, *sema1a.2*, and *plexA* are also expressed in the adult brain in this species. Furthermore, all three *semas* are expressed in the adult MBs, with *sema1a.2* and *sema2a* being most obviously expressed in the Kenyon cell region. The MBs in invertebrates are responsible for multimodal sensory integration, and are essential to learning and memory (Pascual & Preat, 2001). The main cell in the MB is the Kenyon cell, and in many insects, including crickets, Kenyon cells are continuously derived from neuroblasts located at the tip of the MBs through adulthood (Simões & Rhiner, 2017). ISH and immunohistochemistry experiments on *G. bimaculatus* brain tissue sections show that this neurogenic region is surrounded by cells expressing Sema2a (Maynard et al., 2007). Although we did not examine sectioned brain tissue in this study, the ring-like expression pattern of *sema1a.2* was similar to that observed for sema2a, indicating that it may also have a functional role to play in the neurogenic region. In addition, the subcellular expression patterns of *sema1a.2* was similar to what we have previously reported for *sema2a* (Maynard et al., 2007), with staining evident only at the margins of the cell bodies (Figure 6j). Kenyon cell nuclei are reported to be 6 μm in diameter (Cayre et al., 1996), evidently taking up much of the intracellular space in Kenyon cell soma. It is plausible that newborn Kenyon cells in the adult MBs might rely on the continued expression of Semas and Plexins to repel new axons out of the MB and aid in the elaboration of dendritic arbors into the proper locations. Further, these newborn neurons are likely important for the proper function of the MBs, since suppressing neurogenesis with radiation leads to learning and memory deficits in crickets (Scotto-Lomassese et al., 2003).

Given the expression of both *sema1a*-type transcripts in the adult thoracic ganglia, we were surprised to see no appreciable *plexA* expression here. In invertebrates, however, Sema signaling may not always require Plex receptors; in *D. melanogaster* Sema1a can work in "reverse," by acting as a receptor for Plexin, Sema2a, or Sema2b (Godenschwege, Hu, Shan-Crofts, Goodman, & Murphey, 2002; Hernandez-Fleming et al., 2017; Yu, Zhou, Cheng, & Rao, 2010). When Sema1a is bound by a ligand, the cytoplasmic domain interacts with a number of different factors that influence GTPases, which in turn alter axon growth (Cho, Chak, Andreone, Wooley, & Kolodkin, 2012; Hsieh, Chang, Yu, & Rao, 2014; Jeong, Juhaszova, & Kolodkin, 2012). In cricket thoracic ganglia, both *sema1a.2* and *sema2a* were expressed at reasonably high levels. *sema1a.1* was only weakly expressed and *plexA* expression was not detectable. This combination of expression patterns would predict that reverse signaling, in which Sema2a could act as a ligand for a Sema1a.2 receptor, might be more prevalent than forward signaling in the adult thoracic ganglia. It is not clear what necessary functions reverse signaling, rather than the canonical forward signaling, would bring to the ganglion, but it may allow flexibility in the adult tissue beyond what the canonical pathway provides. Experiments in which Sema signaling components are knocked down in embryos and adults will help us understand what role these proteins play in the development and maintenance of the nervous system in *G. bimaculatus*.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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