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Molecular and functional analysis of *Drosophila single-minded* larval central brain expression

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ABSTRACT

Developmental regulatory proteins are commonly utilized in multiple cell types throughout development. The Drosophila single-minded (sim) gene acts as master regulator of embryonic CNS midline cell development and transcription. However, it is also expressed in the brain during larval development. In this paper, we demonstrate that *sim* is expressed in three clusters of anterior central brain neurons: DAMv1/2, BAmas1/2, and TRdm and in three clusters of posterior central brain neurons: a subset of DPM neurons, and two previously unidentified clusters, which we term PLSC and PSC. In addition, sim is expressed in the lamina and medulla of the optic lobes. MARCM studies confirm that sim is expressed at high levels in neurons but is low or absent in neuroblasts (NBs) and ganglion mother cell (GMC) precursors. In the anterior brain, sim⁺ neurons are detected in 1st and 2nd instar larvae but rapidly increase in number during the 3rd instar stage. To understand the regulation of sim brain transcription, 12 fragments encompassing 5'-flanking, intronic, and 3'-flanking regions were tested for the presence of enhancers that drive brain expression of a reporter gene. Three of these fragments drove expression in *sim*⁺ brain cells, including all sim⁺ neuronal clusters in the central brain and optic lobes. One fragment upstream of sim is autoregulatory and is expressed in all sim⁺ brain cells. One intronic fragment drives expression in only the PSC and laminar neurons. Another downstream intronic fragment drives expression in all sim⁺ brain neurons, except the PSC and lamina. Thus, together these two enhancers drive expression in all sim⁺ brain neurons. Sequence analysis of existing sim mutant alleles identified three likely null alleles to utilize in MARCM experiments to examine sim brain function. Mutant clones of DAMv1/2 neurons revealed a consistent axonal fasciculation defect. Thus, unlike the embryonic roles of sim that control CNS midline neuron and glial formation and differentiation, postembryonic sim, instead, controls aspects of axon guidance in the brain. This resembles the roles of vertebrate sim that have an early role in neuronal migration and a later role in axonogenesis.

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The formation of functional central nervous system (CNS) neural circuits consists of a series of events beginning with neurogenesis, followed by axonogenesis, synaptic connectivity, and differentiation. These circuits underlie the complex behaviors found throughout the animal kingdom. One key aspect of CNS development is the action of transcriptional regulatory proteins. Since their number is relatively modest, they are often used multiple times during development to regulate different sets of genes and developmental processes. Mechanistically understanding how transcriptional regulation controls neurodevelopment will ultimately provide insight into the evolutionary basis for species differences in neural circuitry and behavior.

The Drosophila and mammalian single-minded (sim) genes provide an excellent example of how transcription factors perform multiple roles during CNS development (Crews, 2003). Drosophila sim encodes a basic-helix-loop-helix-PAS (bHLH-PAS) protein that forms a DNA binding heterodimer with the Tango (Tgo) bHLH-PAS protein (Sonnenfeld et al., 1997). During embryogenesis, sim is prominently expressed in the cells that lie along the midline of the Drosophila CNS and acts as a master regulator of CNS midline cell development (Nambu et al., 1991). The midline cells consist of a diverse group of motoneurons, interneurons, neurosecretory cells, and glia (Wheeler et al., 2006). In sim mutants, the midline cells fail to form, and midline-specific transcription is generally absent (Nambu et al., 1990). Consistent with a master regulatory role, misexpression of sim throughout the neuroectoderm results in the transformation of the entire CNS into only midline cells (Nambu et al., 1990). Later in development, sim is expressed in differentiated midline glia and neurons and was shown to control midline





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glial transcription (Wharton et al., 1994). During larval development, sim continues to be expressed in the midline cells of the ventral nerve cord and is also expressed in the brain, in both the central brain region and the lamina and medulla of the optic lobes (Pielage et al., 2002). The role of sim in the optic lobes was functionally investigated and shown to play a role in the differentiation of laminar precursor cells into mature neurons (Umetsu et al., 2006). Mammals have two sim genes, Sim1 and Sim2 (reviewed in Fan et al. (1996)). Sim1 is expressed in the developing hypothalamus, including cells of the paraventricular nucleus (PVN), anterior periventricular nucleus (aPV), and supraoptic nucleus (SON). Genetic analysis of Sim1 homozygous mutant mice revealed an absence of the PVN, aPV, and SON and implicated *Sim1* in controlling the migration of PVN and SON neurons into the anterior neuroendocrine hypothalamus (Xu and Fan, 2007). Thus, Drosophila and mammalian sim genes play important roles in generating functional CNS cell types and can control precursor formation. differentiation, and migration.

Genetic and expression data have indicated additional potential roles for both Drosophila and mammalian sim. Drosophila sim is expressed in the larval central brain (Pielage et al., 2002), and in adults this region (central complex) has been implicated in coordinating movement (Strauss, 2002). Behavioral analysis of a temperaturesensitive sim allele revealed that when shifted to the non-permissive temperature after embryonic neurogenesis was complete, adult flies showed locomotor defects (Pielage et al., 2002). Morphological analysis of the adult brain indicated a disorganization of the central complex neuropil. These results suggested a defect in interhemispheric communication and a subsequent inability to coordinate movement. Recent work on murine Sim1 and Sim2 revealed that they play a role in controlling axonogenesis of mammillary body axons (Marion et al., 2005). The results from mammals indicate that sim can function in axonogenesis, and this is also a potential role for sim in central brain development given the sim disorganized neuropil phenotype.

In this paper, we further explore the expression and function of sim in the larval central brain. We demonstrate that sim is expressed in six regions in the central brain, three on the anterior side and three on the posterior side, and identify the relevant neuronal clusters with respect to existing Drosophila brain maps. Many of the sim⁺ neurons send their axons across the midline. Experiments involving a transgenic locus-wide survey of sim DNA fragments identified enhancers that can drive all aspects of *sim* larval brain gene expression. These include distinct enhancers that can drive expression in specific sim⁺ neuronal clusters, as well as a sim autoregulatory enhancer. We utilize sim mutant MARCM clones to show that the loss of sim in an anterior brain cluster results in a defect in the fasciculation of axons that are crossing the midline. This type of defect may result in deficiencies in interhemispheric communication, consistent with sim mutant defects in coordinating movement. These results also reveal further similarities in the function of Drosophila and mammalian sim genes, since both contribute to axonogenesis as well as neurogenesis and migration.

1. Results

1.1. Postembryonic sim⁺ cells predominantly utilize a single transcript

Previous sequence analysis of embryonic *sim* cDNA clones revealed that the *sim* transcription unit consists of nine exons and three different embryonic transcripts (RA, RB, and RC) and spans 20.5 kb of genomic DNA (Fig. 1A) (Kasai et al., 1998). Recent RNA-Seq data further suggests that female adult *sim* expression may utilize an additional, previously unknown exon (exon 2 in Fig. 1A) and

promoter to yield a fourth *sim* transcript (Graveley et al., 2011). The *sim* RA, RB, and RC transcripts utilize two promoters: an early promoter (P_E) and a late promoter (P_L). The RA transcript consists of exons 3–10 and is transcribed from P_E . Transcripts RB and RC contain exons 1 and 4–10. They are transcribed from P_L . The RB and RC transcripts differ in the use of alternative donor splice sites in exon 1. Since exon 1 encodes only 5'-UTR sequences, RB and RC produce the same protein. The entire protein coding sequence of RB and RC is contained within RA, but RA has a different 5'-UTR and an additional 24 aa of coding sequence at the N-terminus. The function of the additional RA protein residues is unknown.

To examine the utilization of the three different embryonic transcripts (RA, RB, and RC) during development, RT-PCR was carried out using transcript-specific primers with RNA from the embryonic, larval, pupal, and adult stages (Fig. 1B). RA is present in only the 0–3 and 3–6 h embryonic collections. The RB transcript is utilized from 3 to 15 h of embryonic development but not later. The RC transcript is observed weakly at 0–3 h but then strongly at all stages of embryonic and postembryonic development. These results closely match Northern blot and RNA-Seq experiments (Crews et al., 1988; Graveley et al., 2011). *sim* is expressed in a variety of embryonic and postembryonic cell types – while different embryonic cell types may express RA, RB, RC, or multiple isoforms, the RT-PCR results suggest that the majority of postembryonic cell types express only RC.

1.2. Identification of sim⁺ neurons in the larval brain

Clusters of sim⁺ neurons (Pielage et al., 2002) in the anterior brain are present in 1st, 2nd, and 3rd instar larvae, and their number significantly increases by the 3rd instar larval stage (Fig. 1C–E). These cells include three paired clusters of neurons on the anterior side of the brain (Fig. 1C-E), neurons on the posterior of the brain (Fig. 1F), and the medulla and lamina of the optic lobes (Fig. 1E). Recent work has provided a comprehensive description of the organization of the 3rd instar larval brain (Pereanu and Hartenstein, 2006). Combining anti-Sim staining with either MAb BP106 (anti-Neurotactin) staining or MARCM allowed us to identify the sim⁺ brain cells as described below. The three paired sim⁺ neuronal clusters on the anterior side of the brain correspond to DAMv1/2, BAmas1/2, and TRdm (Figs. 1E and 2A). Along the dorsal and posterior sides of the brain, there are another three groups of *sim*⁺ neurons (Fig. 1F). One group of sim^+ neurons is derived from the DPMm1-3 and DPMpm2 cell lineages (Fig. 1F; referred to collectively as DPM neurons). The remaining two groups are previously undescribed and are tentatively designated as the Posterior Sim⁺ Cluster (PSC) and Posterior Lateral Sim⁺ Cluster (PLSC). Expression of sim is also present in the cells along the midline of the 3rd instar larval ventral nerve cord (Fig. 1G).

1.2.1. DAMv1/2 sim⁺ cells

Co-staining between anti-Sim and MAb BP106 (anti-Neurotactin) and comparison of their location and axonal morphology (Fig. 2A, B, and D) to the data of Pereanu and Hartenstein (2006) indicates that the dorsal-most anterior sim^+ cells are DAMv1,2 neurons. In addition, using MARCM, we obtained a number of clones for these sim^+ cells that revealed axonal processes characteristic of the DAMv1/2 cells (Fig. 2E). The DAMv1 and DAMv2 cells are derived from two Type I NBs whose progeny are adjacent (Pereanu and Hartenstein, 2006) (Fig. 2B). The axonal processes of each NB fasciculate together and then combine into a single tract that migrates in a posteriormedial direction and crosses the midline in the dorsal anterior commissure (DAC1) (Fig. 2D and E). All of the neuronal progeny of each NB are sim^+ , although co-staining MARCM clones (Lee and Luo, 1999) for Elav, a neuronal marker (Berger et al., 2007), indicates that the NB and GMC are sim^- (Supp.



Fig. 1. Postembryonic CNS expression of sim. (A) Schematic of the sim locus shows the embryonic alternative splice variants of sim: RA, RB, and RC. The sim transcription unit (blue; 20.5 kb) consists of 10 exons, and embryonic expression derives from two promoters: PL and PE. The coding sequence of each transcript is filled tan, and the untranslated regions are filled gray. modENCODE RNA-Seq data (Graveley et al., 2011) revealed a novel female-specific adult exon (F; exon 2), which is likely transcribed from a third promoter. (B) Developmental profile of sim splice variants determined by RT-PCR of total RNA. Multiplex RT-PCR was employed using total RNA from each developmental stage and primers specific for each splice variant and the *RpL32* gene, which is uniformly expressed throughout development (Graveley et al., 2011; O'Connell and Rosbash, 1984). Times of embryonic RNA indicates the collection times in hr after egg laying. Larval RNA was prepared from 1st instar (L1), 2nd instar (L2), 3rd instar (L3) larvae, and L3 brain. Pupal RNA was isolated from light pupae representing the first two days of pupation and dark pupae from the third and fourth days of pupation. Adult collections were from 2-day-old adults (equal numbers of males and females), 2-day-old adult heads, and 2-day-old adult thoraces and abdomens (T + A). No PCR products were observed in the negative controls (NC): (1) PCR without template (-Temp) and (2) PCR without reverse transcriptase (RT). M lanes are molecular weight markers, which confirmed that the RT-PCR products were the expected size. (C-E) Dissected brains stained with anti-Sim from (C) 1st instar (L1), (D) 2nd instar (L2), and (E) 3rd instar (L3) larvae show Sim localization (magenta) in three paired clusters of cells in the anterior central brain throughout larval development. The number of Sim⁺ central brain cells is relatively similar in 1st and 2nd instar larvae but increases significantly between the 2nd instar and late 3rd instar larval stages. The Sim* anterior central brain cells are: (DA) DAMv1/2, (BA) BAmas1/2, and (TR) TRdm neurons. Sim is also present in the lamina (La) and medulla (Me) of the optic lobes. (F) On the posterior side of the brain, Sim is present in neurons in the DPMm1-3 and DPMpm1/2 neurons (DPM), as well as two previously undescribed clusters of neurons that we tentatively refer to as (PSC) Posterior Sim⁺ Cluster and (PLSC) Posterior Sim⁺ Lateral Cluster. (F') Enlarged view of (F) with each neuronal group outlined with a white dotted line. (G) Sim is present in the midline cells (ML) of the 3rd instar larval ventral nerve cord (VNC).



Fig. 2. Identification of *sim*⁺ larval brain neurons. (A) 3rd instar larval brain stained with anti-Sim to show the three paired clusters of *sim*⁺ neurons: (DA) DAMv1/2, (BA) BAmas1/2, and (TR) TRdm neurons. Esophageal opening (Es) lies between the brain hemispheres. (B, C) Brains stained with anti-Sim (magenta) and MAb BP106 (green) showing (B) DAMv1/2 neurons and (C) BAmas1/2 neurons. Each cluster consists of two groups of neurons that extend an axon (arrowheads) that converge into a single axon tract. (D, D') Brain stained with anti-Sim (magenta) and MAb BP106 (green) showing neuronal cell bodies and axon tracts. The characteristic tracts of the DAMv1/2 (DA) and BAmas1/2 (BA) neurons are shown (arrows). The DAMv1/2 axons can be seen crossing the midline in the DAC1 axon tract (arrowhead). (E, E') Brain visualized for GFP (green) and Sim (magenta) showing two DAMv1/2 (DA) MARCM clones that fasciculate together in DAC1 (arrowhead). (F, F') The characteristic ascending MeBd axon tract (arrow) is shown emanating from a BAmas1/2 (BA) MARCM clone. (G, G') Brain stained with MAb BP106 (green) and anti-Sim (magenta) illustrating that the *sim*⁺ TRdm neurons extend a characteristic short projection (arrow) toward the neuropil near the ventral esophagus (Es).

Fig. 1). In one fortuitous example, DAMv1/2 cells were MARCMlabeled on both sides of the brain, clearly showing their axons fasciculating together across the midline (Fig. 2E).

1.2.2. BAmas1/2 sim⁺ neurons

The medial anterior group of sim^+ cells (Figs. 1E and 2A) is the BAmas1/2 neurons. These cells were distinguishable based on their position relative to MAb BP106 staining (Fig. 2C and D). The BAmas1/2 neurons are derived from two Type I NBs, and their axons fasciculate together into a tract that rises vertically within the MeBd tract and then crosses the midline in the DAC1 tract. MARCM clones also confirmed the identity of these cells based on their characteristic axonal trajectories (Fig. 2F). All of the BAmas1/2 neurons are *sim*⁺.

1.2.3. TRdm neurons

The smallest and ventral-most of the anterior sim^+ clusters are the TRdm neurons that form part of the tritocerebrum. These cells lie at the ventral base of the esophageal opening (Figs. 1E and 2A) and can be recognized based on their position and characteristic neurite bifurcation (Pereanu and Hartenstein, 2006) as visualized with MAb BP106 staining (Fig. 2G). All of the TRdm neurons are sim^+ .

1.2.4. DPMm1-3 and DPMpm2 neurons

The dorsal-most *sim*⁺ cells are members of the DPM group of neurons (Pereanu and Hartenstein, 2006). These cells are derived from Type II NBs and are dispersed throughout the dorso-posterior brain and cross the midline via a number of commissural tracts



Fig. 3. Transgenic analysis of the *sim* regulatory region. (A) Schematic of a 37.8 kb genomic region that includes the *sim* gene and neighboring *pic*, *CG*43063, and *timeout* genes. Two insulator protein binding sites surrounding *sim* are indicated by vertical lines. Fragments that were analyzed by *Gal4* transgenesis are labeled A–K and include the fragment size in kb. The 3.*Tsim* fragment has been previously described (Nambu et al., 1991). Green boxes indicate fragments that drive postembryonic expression, while fragments with no postembryonic expression are unfilled. (B, B') *A1.0-GFP.nls* 3rd instar larval brains were stained with anti-GFP (green) and anti-Sim (magenta) to assess whether GFP co-localizes with Sim⁺ brain cells. On the anterior side of the brain, *A1.0-GFP.nls* drives expression in DAMv1/2 (DA), BAmas1/2 (BA), and TRdm (TR). In addition, *GFP* expression is detected in two optic lobe ganglia, the lamina (La) and medulla (Me). (C, C') On the posterior side of the brain, *A1.0-GFP.nls* drives GFP expression in the midline cells (ML) of the larval ventral nerve cord cells. (E, F) Mutant version of *A1.0-GFP.nls*, in which both Sim:Tgo binding sites were mutated, was analyzed. The larval brain was stained for GFP (green) and MAb BP106 (magenta). (E) On the anterior side of the brain, the BP106 staining indicates the location of the DAMv1/2 (DA), BAmas1/2 (BA), and TRdm (TR) neurons, and these cells were GFP⁻. (F) On the posterior side of the same brain, there was an absence of GFP in brain neurons. (C, H) Sagittal views of stage 11 *A1.0-GFP.nls* embryos with (G) unmutated *A1.0-GFP.nls* and (H) a version of *A1.0-GFP.nls* with the Sim:Tgo sites mutated. Single segment is shown with internal up and anterior to the left. Embryos are stained for GFP (green) and anti-Sim (magenta), which stains all midline cells. The unmutated *A1.0-GFP.nls* drives robust expression in all midline cells, whereas expression is absent in the mutated version.

(Izergina et al., 2009). *sim* is expressed in only a subset of DPM neurons. The identification of the sim^+ subset of DPMm1–3 and DPMpm2 neurons is based on their location in the brain (Fig. 1F).

1.2.5. PSC and PLSC neurons

This small cluster of *sim*⁺ cells, tentatively named PSC, was previously unidentified, most likely because they stain poorly with MAb BP106 in the 3rd instar larvae. The lack of BP106 staining indicates that these are primary neurons, and not secondary neurons (Pereanu and Hartenstein, 2006). When *Gal4* lines expressed in the PSC (see below) are crossed to UAS-tau-GFP, an occasional neurite is observed that crosses the midline (Fig. 4E). The PLSC group of sim^+ neurons is slightly ventro-lateral to the PSC cells and consists of more neurons. These cells are located more posterior than lineages previously described.

1.3. Transgenic screen for postembryonic sim-specific brain enhancers

Identifying regulatory DNA fragments that drive expression in specific cells corresponding to the gene's normal expression



Fig. 4. Two distant fragments recapitulate central brain expression. (A) Schematic depicting the location of the B2.4 and F1.4 fragments with respect to the *sim* gene. B2.4 is located within the first intron 10.4 kb away from the F1.4 fragment, which spans the entire third intron. (B, B') *B2.4-Gal4 UAS-nuc-GFP* drives GFP expression in the PSC neurons (arrow) on the posterior side of the larval brain. Brains stained with anti-GFP (green) and anti-Sim (magenta). (C) Staining *B2.4-Gal4 UAS-nuc-GFP* with MAb BP106 and anti-GFP illustrates axon tracts characteristic of the DPMm1-3 and DPMpm2 nerve cell clusters (BP106) and indicates that the PSC neurons reside near the site where some posterior axonal tracts enter the commissure. (D, D') *B2.4-Gal4 UAS-nuc-GFP* drives *GFP* expression in Sim^{*} optic lobe laminar neurons (La). (E) *B2.4-Gal4 UAS-tau-GFP* stained with anti-Tau2 (green) reveals an axonal projection (arrow) emanating from the PSC that crosses the midline (dotted line) and fasciculates with contralateral PSC axons. (F) *F1.4-Gal4 UAS-mCD8-GFP* reveals *GFP* expression (green) in the Sim^{*} DAMv1/2 (DA), BAmas1/2 (BA), and TRdm (TR) neurons. Brains were stained with anti-GFP and MAb BP106 (magenta). (G, G') On the posterior side of the brain, *F1.4-Gal4 UAS-nuc-GFP* expresses *GFP* (green) in the Sim^{*} DPM neurons and PLSC neurons but not in the PSC neurons (arrows).

pattern can enhance insight into the expression and function of a gene. This is commonly carried out in *Drosophila* using the Gal4/ UAS transgenic approach (Brand and Perrimon, 1993) in which fragments of DNA are coupled to promoter-Gal4, and that transgenic strain is crossed to a reporter (e.g. UAS–GFP) for expression studies or crossed to a UAS transgenic line that allows cell ablation or reduction/enhancement of neural transmission. Our goals were to identify larval brain enhancers to better understand brain gene expression and to potentially isolate Gal4 strains that could target Gal4 to specific subsets of brain cells for future functional studies.

We initially assayed 12 fragments spanning 29.6 kb of sim genomic DNA, including 1.2 kb 5' to sim, introns 1, 3, and 7 (with respect to RC) and 9.6 kb 3' to sim (Fig. 3A). The piccolo (pic) gene is 1.0 kb 5' to sim, and the timeout gene lies 10.4 kb 3' to sim. Thus, the extent of the sim gene is potentially 31.9 kb, assuming that no sim regulatory elements reside within or distal to pic and timeout. Fragments were PCR-amplified, cloned into the pBCGw-UCP PhiC31 Gal4 vector, and introduced into the attP2 site at 68A4 using germline transformation (Groth et al., 2004), with the exception of the existing sim3.7 Gal4 line (Nambu et al., 1991). These fragments were assayed in vivo for their ability to drive postembryonic larval CNS expression. Immunostaining with anti-Sim was used to identify *sim*⁺ cells. As described below, we have identified enhancers that drive GFP in all known sim⁺ postembryonic cell types, and thus all *sim* postembryonic enhancers may reside within the 31.9 kb sim locus.

1.4. The A1.0 fragment is an autoregulatory enhancer that drives expression in all postembryonic sim⁺ cells

The A1.0 fragment contains P_L and 1.0 kb of 5'-flanking genomic DNA. Previous work had shown that this region contained an enhancer that drove embryonic midline expression (Muralidhar et al., 1993), and this expression was genetically dependent on *sim* (Nambu et al., 1991). We confirmed that *A1.0-GFP.nls* drives embryonic midline expression (Fig. 3G). When postembryonic expression was assayed, we observed that *GFP* reporter expression was present in all *sim*⁺ brain cells and the midline cells of the ventral nerve cord. This included the *sim*⁺ anterior brain neurons (Fig. 3B), the medulla and lamina of the optic lobes (Fig. 3B), the posterior brain neurons (Fig. 3C), and larval VNC midline cells (Fig. 3D) (Table 1).

The Sim:Tgo heterodimeric transcription factor binds to ACGTG sequences (Sonnenfeld et al., 1997). The A1.0 fragment has two ACGTG putative Sim:Tgo binding sites. When these sites were mutated together in *A1.0-Gal4* and tested after germline line transformation, all embryonic midline and postembryonic expression was absent (Fig. 3E, F, and H). This is consistent with a requirement for *sim* autoregulation in A1.0-driven CNS and midline expression. However, it also suggests that the subdivision of A1.0 is unlikely to lead to transgenic lines that drive expression in subsets of *sim*⁺ brain cells, and no further subdivision of A1.0 was undertaken.

1.5. Two complementary sim midline enhancers recapitulate sim expression in the central brain and optic lobes

Two fragments, B2.4 and F1.4, each drive gene expression in non-overlapping subsets of sim^+ central brain and optic lobe neurons. B2.4 expression is present in the PSC and lamina of the optic lobes (Fig. 4A–E). Crossing *B2.4-Gal4* to *UAS-tau-GFP* reveals an outgrowth of axons from the PSC neurons that project dorsally and then cross the midline (Fig. 4E). The F1.4 fragment drives expression in the central brain in all sim^+ anterior and posterior clusters, except PSC neurons (Fig. 4F and G). In addition, F1.4 drives expression in the medulla of the optic lobes (see below). Thus, the B2.4 and F1.4 fragments contain enhancers that together control the expression of all *sim* brain expression; they are complementary and control non-overlapping aspects of *sim* expression (Table 1).

1.6. Molecular dissection of distinct sim⁺ brain enhancers

One issue concerning how sim postembryonic brain expression is controlled is the nature of the brain enhancers: is expression of each sim^+ neuronal cluster driven by a separate enhancer, or do individual enhancers drive expression in multiple clusters? To begin to address this question, the F1.4 fragment was divided into four subfragments, and each was tested for brain expression (Fig. 5A and Table 1). The L1.0 fragment drives expression only in the sim⁺ DPM, PLSC, and medulla neurons (Fig. 5B) but not in the anterior brain neurons. The adjacent M582 fragment is expressed in only the medulla (Fig. 5C). The number of medullary neurons is reduced compared to F1.4. Although L1.0 and M582 together include the complete F1.4 fragment, which is expressed in the anterior brain neurons, neither L1.0 nor M582 drives expression in these cells. However, one L1.0 subfragment, N494, drives expression in the DAMv1/2 and BAmas1/2 neurons but not the TRdm neurons (Fig. 5D), so that anterior brain enhancers reside in L1.0. On the posterior side of the brain, N494 drives expression in the DPM and PLSC neurons (Fig. 5E), and in a subset of medulla neurons. The O501 fragment has only non-specific expression in scattered brain cells (Fig. 5F and G). Thus, the N494 fragment has enhancers for all of the sim⁺ brain clusters found in F1.4, expect TRdm, which was not expressed by any of the F1.4 subfragments. There are two distinct enhancers that contribute to medulla expression: L494 and M582. Oddly, the L1.0 fragment, which encompasses N494, only drives posterior, but not anterior, brain expression.

Table 1

Summary of *sim* transgenic line larval CNS expression. A summary of transgenic lines that drive reporter gene expression in *sim*⁺ neurons in the larval brain. An "X" indicates expression in a particular cell type, while a blank square signifies absence of expression.

Transgene	DAMv1/2 BAmas1/2	TRdm	DPM	PLSC	PSC	Optic lobe lamina	Optic lobe medulla	VNC midline	Pan-neural	Non-Sim⁺ Brain	None
A1.0-GFP	Х	Х	х	х	Х	Х	Х	Х			
B2.4-Gal4					Х	Х					
C2.3-Gal4									Х		
D2.1-Gal4											Х
E2.3-Gal4											Х
3.7sim-Gal4											Х
F1.4-Gal4	Х	Х	Х	Х			Х				
L1.0-Gal4			Х	Х			Х				
M582-Gal4							Х				
N494-Gal4	Х		Х	Х			Х				
0501-Gal4										Х	
P261-Gal4	Х	Х	Х	Х			Х				
Q255-Gal4		Х	Х				Х				
G1.1-Gal4										Х	
H2.4-Gal4											Х
I2.6-Gal4											Х
J2.5-Gal4										Х	
K2.6-Gal4										Х	



Fig. 5. Intron 3 subfragments drive expression in subsets of Sim⁺ cells. (A) The schematic illustrates subfragments of the F1.4 fragment tested for postembryonic brain expression. Green blocks indicate Gal4 constructs that drive GFP in *sim*⁺ brain cells, and the unfilled block indicates an absence of Sim⁺ brain expression. (B, B') *L1.0-Gal4* drives *UAS-mCD8-GFP* expression in the DPMm1-3, DPMpm2, and PLSC neurons (not shown) on the posterior side of the brain. The brain was stained for GFP (green) and Sim (magenta), and the DPM clusters are circled. (C, C') *M582-Gal4 UAS-nuc-GFP* expression drives GFP expression (green) in a subset of medullary neurons (Me). (D) *N494-Gal4 UAS-tau-GFP* reveals GFP expression (green) in the DAMv1/2 and BAmas1/2 Sim⁺ neurons on the anterior side of the brain. The BAmas1/2 axons project ipsilaterally before crossing the milline in the commissural tract (arrowhead) along with axons from DAMv1/2. (E) GFP⁺ cells with *N494-Gal4 UAS-tau-GFP* expression in the DPM neurons, the GFP-labeled neurons send their axons across the DPC1 commissure (arrowhead). (F, G) 0501-*Gal4 UAS-mCD8-GFP* shows sporadic GFP⁺ cells on both the (F) anterior and (G) posterior sides of the brain that only coincidentally overlap with Sim⁺ neurons.

The N494 fragment, which is expressed in most anterior and posterior Sim⁺ neurons, was further subdivided into two fragments, P261 and Q255 (Fig. 6A and Table 1), to investigate *sim* brain enhancers. Expression driven by the P261 fragment closely resembled N494-driven expression. *P261-Gal4 UAS-nuc-GFP* showed *GFP* expression in all anterior Sim⁺ brain regions (Fig. 6B), the DPM and PLSC posterior brain clusters and the medulla (Fig. 6D). Interestingly, P261 showed *GFP* expression in the TRdm neurons, even though expression was absent in these cells in *N494-Gal4*. The Q255 fragment drove expression in TRdm (Fig. 6C) and weakly in a scattered subset of PLSC (Fig. 6E) and medulla neurons.

In summary, a single 261 bp fragment, P261 that is derived from F1.4, contains enhancers for all sim^+ brain regions, except PSC and the optic lobe lamina. However, the adjacent Q255 fragment also includes elements driving expression in TRdm and weakly in the PLSC and medulla. In addition, another non-overlapping fragment, M582, has elements driving medulla expression. The B2.4 fragment has enhancers that drive PSC and lamina expression, which complements F1.4 expression. The A1.0 fragment has expression in all sim^+ brain cell types and is driven by *sim* autoregulation. Thus, *sim* central brain expression consists of a combination of distinct enhancers that together initiate and maintain brain expression. In some cases (e.g. medulla), there are multiple enhancers that contribute to expression. It also is possible that enhancers driving expression in some sim^+ cells (e.g. DAMv1/2 and BAmas1,2) are not separable.

1.7. Identification of a sim pan-neuronal enhancer

Additional fragments had varying patterns of expression that did not match known *sim* expression. Three fragments (G1.1, J2.5, and K2.6; Supp. Fig. 2A) drove expression in the brain but not in the cells that overlap with *sim* expression (Supp. Fig. 2B–H); Table 1. Five fragments (D2.1, E2.3, *sim*3.7, H2.4, and I2.6) did not drive relevant brain expression (Table 1). Two of the fragments with expression in brain cells that do not overlap with *sim*, J2.5 and K2.6, reside in



Fig. 6. P261 drives expression in most central brain cells. (A) The N494 fragment from intron 3 was further subdivided into P261 and Q255. (B, D) *P261-Gal4 UAS-nuc-GFP* revealed GFP expression in: (B) all anterior Sim⁺ central brain neurons, including TRdm, and (D) the medulla and all posterior Sim⁺ neurons, except PSC. (C,E) *Q255-Gal4 UAS-nuc-GFP* was expressed in (C, C') TRdm neurons and (E, E') weakly in the PLSC neurons. Brains were stained with anti-GFP (green) and anti-Sim (magenta).

the sim 3'-flanking regions and are the most distal fragments from the sim transcription unit that were tested. These fragments may contribute to the brain expression of the adjacent timeout or CG43063 genes. Another fragment, C2.3 (Supp. Fig. 3A), produces unusual expression in all NBs, GMCs, and neurons in the 3rd instar larval brain (Supp. Fig. 3B). The highest expression is present in NBs and GMCs, while expression in neurons is weaker (Supp. Fig. 3C). Expression is also observed in the optic lobe, although at low levels, and in the ventral nerve cord, with the exception of the posterior-most abdominal segments. Multiple overlapping transformants employing different promoters and vectors all showed the same pattern, indicating that the expression was not due to a cloning artifact. While C2.3 pan-neuronal expression overlaps with Sim⁺ neurons in the brain, the C2.3 pattern is highly distinct from sim, and the C2.3 enhancer presumably reflects the existence of a panneuronal enhancer normally repressed in the CNS.

1.8. sim functions in controlling central brain axon guidance

To assess the genetic role of *sim* in central brain development, MARCM (Lee and Luo, 1999) was carried out with multiple *sim* alleles to generate mutant clones. Since *sim* mutant strains with severe embryonic phenotypes had not previously been sequenced nor their corresponding molecular defects identified, we sequenced all seven coding sequence exons of 12 *sim* alleles (Supp. Fig. 4A, Supp. Table 1; another *sim* allele, *sim*^{11–47}, was sequenced previously (Pielage et al., 2002)). Three mutants (*sim*², *sim*⁸, and *sim*^{BB68}) predicted to produce truncated proteins were selected for MARCM, since they are likely to be amorphic. Mutant *sim* embryos from the three selected strains were stained with anti-Sim raised against a bacterially synthesized protein fragment that is predicted to lack all three mutant proteins, and no Sim immunoreactivity was observed in homozygous mutant embryos (Supp. Fig. 4B). In addition, all three showed severe *sim* collapsed axon scaffold phenotypes (Thomas et al., 1988).

Mutant clones were analyzed for the DAMv1/2 neurons, since they could be readily identified based on their location adjacent to Sim⁺ neurons from the adjoining DAMv1/2 NB progeny and they have observable axonal projections in the 3rd instar larval brain (Fig. 7). As a control, we analyzed MARCM larvae that did not harbor a mutant *sim* allele (Fig. 7A and B). Each wild-type and *sim* clone possessed a NB, associated GMCs, and Elav⁺ neurons. Clones mutant for *sim* had similar numbers of neurons ($N = 41.4 \pm 5.2$, n = 10) (Fig. 7C–G) as wild-type clones ($N = 36.0 \pm 4.0$, n = 8) (Fig. 7A and B). Each wild-type and mutant clone contained ~50% of the DAMv1/2 neurons, indicating that most of the progeny of one of the two DAMv1/2 NBs were GFP⁺. Thus, in the case of the *sim* mutant clones, most, if not all, of the neurons from a single NB were mutant. These results indicate that the role of *sim* in DAMv1/2 neurons does not greatly affect neurogenesis, unlike the role of *sim* in controlling embryonic CNS midline cell formation (Crews, 2003). Instead, axonal fasciculation defects were apparent in *sim* larval brain mutant MARCM clones.

All wild-type MARCM clones within the DAMv1/2 cells (n = 8) extended axons with a similar morphology (Fig. 7A' and B'). The GFP⁺ axons leave the soma dorsally as a common fascicle, elaborate filopodia ipsilaterally (Pereanu and Hartenstein, 2006), and make a sharp turn toward the contralateral side via the supraesophageal commissure. In the commissure, contralateral Sim⁺ axons form a common tract. MARCM *sim* mutant clones often showed axon defects (Fig. 7C'-G'). Of the 12 *sim* mutant DAMv1/2 MARCM clones,



Fig. 7. *Sim* mutant clones show axon fasciculation defects. Wild-type and mutant MARCM GFP⁺ (green) DMAv1/2 clones were stained with anti-Sim (magenta). For each pair of images, the left panel shows a single optical slice showing the GFP⁺ cell bodies, and the right panel is a projection that illustrates the axonal morphology. (A, B) Two wild-type GFP⁺ clones that overlap Sim⁺ neurons. The NB and GMC are Sim⁻. (A', B') The characteristic DAMv1/2 axon tract is apparent and extends centro-dorsally toward the neuropil, then elaborates filopodia (arrow) before projecting contralaterally (arrowhead) across the supraesophageal commissure. (C, D) Two *sim^{BB68}* mutant clones that are adjacent to DMAv1/2 Sim⁺ neurons (arrowheads). (C', D') The axons split into multiple fascicles (arrowheads) rather than traverse the supraesophageal commissure as a single, tight fascicle. (E–F') Two *sim²* clones and a (G, G') *sim⁸* clone that also show multiple branches.

seven showed a mutant phenotype, and five appeared wild type. All three alleles had at least one clone with a mutant phenotype, and all alleles showed a similar defect. In general, the axons of *sim* mutant clones extended a tract centro-dorsally, formed an ipsilateral filopodial protuberance, and then turned toward the supraesophageal commissure, resembling wild-type axons. However, a subset of mutant axons aberrantly defasciculated from the main bundle and continued across the midline. While the phenotypes differed in individual clones, they consistently revealed fasciculation defects. In summary, the mutant clones of all three *sim* alleles showed similar axon fasciculation defects, while neurogenesis was unaffected.

2. Discussion

2.1. The Drosophila sim gene is expressed in multiple clusters of identifiable larval brain neurons

Previously, we demonstrated that *sim* is expressed in three anterior clusters of neurons in the 3rd instar larval brain. In this paper, we show that these three clusters are the DAMv1/2, BAmas1/2, and TRdm neurons. Neurons that are Sim⁺ are present in 1st, 2nd, and 3rd instar larvae at three discrete positions in the anterior brain (Fig. 1A–C). It is likely that the cells in 1st and 2nd instar larvae correspond to the identified 3rd instar clusters, but this has not been directly shown. All five NBs that give rise to these neurons are Class I NBs, in which the NB generates GMCs, each of which divides once to give rise to two neurons. Axons of the DMAv1/2 and BAmas1/2 neurons ultimately cross the midline, whereas the TRdm axons project ipsilaterally and do not cross. The specific larval and adult functions of these neurons are unknown.

In this paper, we describe three additional clusters of Sim⁺ neurons that reside on the dorsal/posterior side of the brain. The dorsalmost cluster can be identified as a subset of DPM neurons, which are derived from a Type II NB that generates transit-amplifying progenitors. Another cluster of neurons could not be unambiguously identified, and we tentatively name it PLSC. These cells are also likely to be derived from Type II NBs, since they are relatively dispersed. The third cluster is a small group of neurons, which also were not previously identified, and are tentatively named PSC. The Sim⁺ DPM, PLSC, and PSC neurons all send axonal projections across the midline.

2.2. sim mutant DAMv1/2 neurons have axon fasciculation defects

Previous analysis of the sim^{J1-47} allele showed defects in adult walking behavior (Pielage et al., 2002). These defects were interpreted as an inability to coordinate movement and are consistent with a lack of interhemispheric communication. Consistent with this notion is the occurrence of five clusters of sim⁺ neurons in the central brain that cross the midline and could be communicating information that coordinates movement. Analysis of the sim¹¹⁻ ⁴⁷ adult central complex neuropil revealed a disorganization of the axons that cross the midline (Pielage et al., 2002), providing a cellular rationale for the behavioral defects. However, it is unclear whether this phenotype reflects an absence of sim function or a hypomorphic condition. In addition, it is unknown whether the underlying phenotype affects neurogenesis or axonogenesis, since either could result in the observed adult phenotype. The sim^{J1-47} phenotype may also be due to an indirect non-autonomous effect of sim on other neurons or axons. In this paper, we used MARCM to examine the sim null mutant phenotype in DAMv1/2 neurons. After sequencing exonic DNA from 13 sim mutants, we selected three that were derived from different genetic backgrounds and are likely to be null mutants. All three mutants possess in-frame stop codons that should produce truncated Sim proteins. *sim*⁸ is predicted to produce a protein only 12 amino acids long, whereas *sim*² and *sim*^{BB68} should produce proteins that terminate in the PAS-2 domain and lack Sim activation domains. Previous work has shown that the absence of the *sim* activation domains results in a protein that cannot activate transcription *in vivo* (Estes et al., 2001; Franks and Crews, 1994). Consistent with these molecular defects, all three mutants showed similar defects. In all mutant clones, the neurons and initial axonal projections appeared normal, indicating no obvious effects of *sim* on neurogenesis and neurite outgrowth. However, in 7/12 mutant clones, there were clear axon fasciculation defects. In wild-type clones, the axons from all DAMv1/2 neurons extended across the midline as a single, tightly fasciculated bundle, whereas in *sim* mutant larvae there were more than one bundle and the axons appeared frayed.

While provocative, the *sim* axonal and behavioral phenotypes raise a number of issues. The first is that while axon guidance defects are observed for the DAMv1/2 neurons, it is unknown whether the behavioral defects are the result of this defect, since the sim¹¹⁻⁴⁷ mutation may also affect sim function in the other anterior and posterior Sim⁺ brain neurons, the optic lobes, and the midline cells of the ventral nerve cord. It is also is possible that reductions in sim could control additional aspects of terminal differentiation and neurotransmission, which could also contribute to the behavioral phenotype. Another potential developmental role is that the *sim*⁺ cells themselves do not physiologically contribute to locomotion, but their axons may pioneer the axons of other neurons that control movement. Finally, since only about half of the cells in each cluster were mutant, the presence of genetically wild-type axons mixed with sim mutant axons could mask the severity of the phenotype. These issues can ultimately be resolved using targeted expression of various transgenes affecting sim function, differentiation, and neurotransmission. Axon guidance defects can be assayed in the other sim^+ neurons using MARCM, but behavioral phenotypes will need to be addressed by targeting disruptions of sim function specifically to each neuronal cluster. The ability to do this was one of the goals of this study, although the Gal4 lines we generated still generally lack sufficient specificity to fully address this issue. Nevertheless, what is clear is that sim controls proper axonal patterning, but not neurogenesis, of the sim⁺ DAMv1/2 neurons.

Mammalian Sim1 and Sim2 and Drosophila sim play multiple roles in development, both in the CNS and in other cell types (Crews, 2003; Fan, 2003). Within the CNS, each plays a role in neurogenesis or neural migration and later in axonogenesis. Drosophila sim controls neurogenesis of embryonic CNS midline cells (Nambu et al., 1991) and differentiation of the optic lobe laminar neurons (Umetsu et al., 2006). In mammals, Sim1 plays a prominent role in neuronal migration in the hypothalamus (Xu and Fan, 2007). Additionally, the murine Sim1 and Sim2 genes are expressed in the mammillary body and control axonogenesis (Marion et al., 2005). In wild-type mice, the Sim1⁺ Sim2⁺ mammillary body cells extend axons along the principal mammillary tract (PMT) that project to the thalamus and tegmentum via the mammillotegmental (MTEG) and mammillothalamic (MTT) tracts. Genetic experiments indicated that the MTEG and MTT are greatly reduced in Sim1 Sim2 double mutant embryos and, to a lesser degree, in Sim1 single mutant embryos. Normally, the PMT extends along the ipsilateral side of the developing brain, but in Sim1 Sim2 mutant embryos, the axons abnormally cross the midline. This suggests that the mammillary body axons no longer respond to a midline-directed repellent in Sim mutant embryos. Consistent with this interpretation, Sim was shown to normally repress expression of Rig-1/Robo3, a gene that antagonizes Slit-mediated repulsion (Marillat et al., 2004; Sabatier et al., 2004). Consequently, upregulation of Rig-1/Robo3 in Sim mutant embryos results in the loss of PMT repulsion by the midline.

The Drosophila sim DAMv1/2 axonal defect differs from the mammalian Sim mutant defect in that the DAMv1/2 axons show fasciculation defects. Significantly, targeting appears roughly correct, since mutant axons branch and migrate toward the midline. Presumably, sim regulates the expression of one or more genes involved in controlling axonal fasciculation, although the identities of those genes are unknown. There are a number of *Drosophila* cell adhesion proteins that have been implicated in axon fasciculation (Van Vactor, 1998), including Fasciclin II, Roughest, and Cadherin-N. One possible explanation for the *sim* phenotype is that Sim positively regulates the levels of cell adhesion/fasciculation proteins, and when their levels drop below a certain point, defasciculation can occur. Alternatively, there exists a class of genes that are anti-adhesive, such as beaten path (Fambrough and Goodman, 1996) and protein tyrosine phosphatases (Desai et al., 1996), which may normally be repressed or silenced by Sim, and, thus, in sim mutants become active and promote defasciculation. Further insights into the mechanisms that govern axon guidance of Sim⁺ cells will require identifying the relevant transcriptional targets of Sim.

2.3. Multiple enhancers control sim postembryonic expression

Comprehensive transgenic analysis of the *sim* regulatory region identified enhancers for all postembryonic sim⁺ brain neurons (Table 1). The P261 fragment drives expression in the anterior central brain, DPM, PLSC, and medulla neurons. The B2.4 fragment drives expression in the PSC and lamina. Thus, these two fragments, which do not overlap in expression, account for all of the central brain and optic lobe expression. In addition to the P261 enhancer. an adjacent fragment, Q255, drives expression in TRdm, DPM, and the medulla, and another proximate fragment, M582, drives medulla expression. Thus, in some cases, there are multiple enhancers that contribute to expression in specific brain neurons. However, we have not yet sufficiently subdivided the *sim* enhancer fragments into subfragments capable of driving the expression of each individual brain cell type. In some cases, this may not be possible if expression in different cell types share transcription factor binding sites. In addition, another enhancer in the A1.0 fragment, which resides in the 5'-flanking sequences, is autoregulatory (Crews and Pearson, 2009) and drives expression in all larval brains cells.

The genomic arrangement of the *sim* brain enhancers provides insights into the mechanisms that control sim expression. RT-PCR data strongly suggest that the brain enhancers function through P₁. While the upstream A1.0 autoregulatory enhancer interacts with P_L in a straightforward manner, the B2.4 and F1.4 enhancers are downstream of P_L and P_E and must skip over P_E to interact with P₁. No relevant brain enhancers were found 3' to the sim transcription unit, although enhancers in fragments J2.5 and K2.6 with brain expression patterns that do not overlap with sim^+ neurons are present in the 3'-flanking region. They may control expression of genes 3' to sim. ChIP-chip embryonic protein analysis of Drosophila insulators by modENCODE has revealed strong binding of the CTCF, Mdg4, and Su(Hw) proteins to a site just 3' of the sim transcription unit (Negre et al., 2010) (Fig. 3A). These proteins may act as insulators to block enhancers 3' to sim from interacting with upstream sim promoters. Similarly, an insulator site just 3' to the pic gene (Fig. 3A) may act to insulate pic enhancers from acting on sim promoters, and vice-versa.

3. Experimental procedures

3.1. Drosophila strains

Thirteen *sim* alleles were analyzed from five sources: *sim*¹, *sim*², *sim*⁵, *sim*⁶, *sim*⁷ (Hilliker et al., 1980); *sim*⁸ (Mayer and

Nusslein-Volhard, 1988), sim^{BB68} , sim^{U22} , sim^{M55} , sim^{TT63} , sim^{W3} (J. Skeath and C. Doe; unpubl.), sim^{I-47} (Pielage et al., 2002), and sim^U (unknown). All strains were tested for noncomplementation with the sim^2 null mutant at 25 °C. Mutant stocks were balanced over *TM3 Sb P[w⁺; Krüppel-Gal4] P[w⁺; UAS-GFP]* (Casso et al., 2000) or *TM3 Sb P[vy⁺; ftz-lacZ]*. The marked balancer chromosomes allowed identification of homozygous mutant embryos. The amorphic alleles sim^2 , sim^8 , and sim^{BB68} were recombined onto a chromosome bearing *FRT*^{82B} for use in MARCM analyses. These chromosomes were combined with *w*; *tub-Gal4 FRT*^{82B} *tub-Gal80/TM3* (J. Treisman) and *elav-Gal4 UAS-mCD8-GFP hs-FLP w* (Bloomington *Drosophila* Stock Center). UAS lines employed for assaying transgenic Gal4 expression included UAS-mCD8-GFP (Lee and Luo, 1999), UAS-tau-GFP (Brand, 1995), and UAS-nuc-GFP (UAS-LacZ::GFP.nls) (Shiga et al., 1996).

3.2. RT-PCR

Total RNA was extracted from 0–3, 3–6, 6–9, 9–12, to 12–15 h (after egg laying, AEL) embryos as well as from 1st, 2nd, and 3rd instar larvae, light (1–2 day) and dark (3–4 day) pupae, and 2-day-old adults using QIAshredder and RNeasy kits (Qiagen). Samples were treated with RQ1 RNase-free DNase (Promega) and converted to single stranded cDNA using SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo-d(T). Transcripts were then detected by PCR in the linear range using the following primers:

sim ForE 5'-TGG ATG CTG GTT GAT GTG CGG-3' sim ForL 5'-CAG GGA TAT GAG CAA GTG CTG AGA A-3' sim Rev 5'-GCC CAA GTG CCA TAA ACG CAA T-3' RpL32 For 5'-ATC CGC CCA GCA TAC AGG-3' RpL32 Rev 5'-CTC GTT CTC TTG AGA ACG CAG-3'

Use of the primer pair, *sim* ForE and Rev, detected transcripts derived from P_E (*sim* RA), whereas use of the primer pair, *sim* ForL and Rev, detected P_L transcripts (*sim* RB, RC). RT-PCR products were sequenced to confirm their identity.

3.3. Transgene construction

DNA fragments containing intragenic and intronic sequences from the *sim* locus were PCR-amplified and subcloned into a spectinomycin-resistant pCR8/GW/TOPO Gateway-compatible vector (Invitrogen) and transformed into *Escherichia coli*. The primer pairs used for PCR amplification are listed in Supp. Table 2. The fragments in pCR8, except A1.0, were then cloned via Gateway LR recombination into an ampicillin-resistant PhiC31 transformation vector, pBPGw-UCP (J. Pearson). The A1.0 fragment was cloned into Mintgate (Jiang et al., 2010) to generate *A1.0-GFP.nls*. Each *sim* transgenic construct was microinjected into *Drosophila* embryos that express germline-localized PhiC31 integrase and contain the PhiC31 genomic destination site attP2 (68A4) (Groth et al., 2004). PCR-based site-directed mutagenesis was used to mutate both A1.0 Sim:Tgo consensus binding sites (NACGTG) to GGATCC. Primers used are listed in Supp. Table 2.

3.4. Immunostaining

Embryos were collected, fixed, and stained using standard procedures (Patel et al., 1987). Larval brains were dissected in 1X PBS, fixed in 4% formaldehyde in $1 \times$ PBS for 20 min, and stained using the following antibodies and reagents: rat anti-Sim (1:100) (Ward et al., 1998), guinea pig anti-Sim (1:200) (Wheeler et al., 2008), murine MAb anti-BP106 (1:100, Developmental Studies Hybridoma Bank, Iowa) (Hortsch et al., 1990), murine anti-Tau2 (1:500; Sigma), rabbit anti- β -galactosidase (1:2000; Cappel), murine MAb 9F8A9 anti-Elav (1:100; Developmental Studies Hybridoma Bank, Iowa) (O'Neill et al., 1994), rabbit anti-GFP (1:1000; Abcam), Cy3-, Cy5-, Alexa350-, Alexa488-, Alexa568-conjugated secondary antibodies (1:1000; Invitrogen), HRP-conjugated secondary antibodies (Jackson ImmunoResearch), and 1.0 mg/ml DAPI (1:1000; Sigma). Fluorescently stained specimens were mounted in Aqua-Poly/Mount (Polysciences) or glycerol and imaged on Zeiss LSM510 and Pascal confocal microscopes using 40× objectives.

3.5. MARCM

hs-Flp elav-Gal4 UAS-mCD8-GFP; tub-Gal4 FRT^{82B} tub-Gal80/+ males were crossed to virgin females bearing either FRT^{82B} sim^2 ; FRT^{82B} sim^8 ; FRT^{82B} sim^{BB68} ; or FRT^{82B} $P[w^+]$ chromosomes (Lee and Luo, 1999). Embryos were collected for 3 h and aged for 24 h at 25 °C, then heat shocked for 1.5 h at 37 °C, followed by aging at 25 °C until they became wandering 3rd instar larvae. Brains were isolated, fixed, stained, and analyzed as described above.

3.6. Sequencing of sim mutant DNA

Genomic DNA was isolated from stage 14 to 15 homozygous *sim* mutant embryos that were identified based on the absence of balancer chromosome *GFP* expression. Sequencing of the *sim* gene was performed using DNA fragments isolated by touchdown PCR (tdPCR) (Don et al., 1991; Hecker and Roux, 1996). Seven sets of primer pairs were used to amplify exons 4–10, which comprise most of the *sim* coding sequence and corresponding splice sites (Supp. Table 3). PCR products were purified and sequenced at the UNC-CH Genome Analysis Facility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gep.2011.09.002.

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