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Heparan Sulfate Sugar Modifications Mediate the Functions of Slits and Other Factors Needed for Mouse Forebrain Commissure Development

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Heparan sulfate proteoglycans are cell surface and secretory proteins that modulate intercellular signaling pathways including Slit/Robo and FGF/FGFR. The heparan sulfate sugar moieties on HSPGs are subject to extensive postsynthetic modification, generating enormous molecular complexity that has been postulated to provide increased diversity in the ability of individual cells to respond to specific signaling molecules. This diversity could help explain how a relatively small number of axon guidance molecules are able to instruct the extremely complex connectivity of the mammalian brain. Consistent with this hypothesis, we previously showed that mutant mice lacking the heparan sulfotransferases (Hsts) Hs2st or Hs6st1 display major axon guidance defects at the developing optic chiasm. Here we further explore the role of these Hsts at the optic chiasm and investigate their function in corpus callosum development. Each Hst is expressed in a distinct pattern and each mutant displays a specific spectrum of axon guidance defects. Particular Hs2st−/− and Hs6st1−/− phenotypes closely match those of Slit1−/− and Slit2−/− embryos respectively, suggesting possible functional relationships. To test functional interactions between Hs2st or Hs6st1 and Slits we examined optic chiasm and corpus callosum phenotypes in a panel of genotypes where Hs2st or Hs6st1 and Slit1 or Slit2 function were simultaneously reduced or absent. We find examples of Hs2st and Hs6st1 having epistatic, synergistic, and antagonistic genetic relationships with Slit1 and/or Slit2 depending on the context. At the corpus callosum we find that Hs6st1 has Slit-independent functions and our data indicate additional roles in FGF signaling.

Introduction

The mammalian brain is composed of an enormous number of neurons forming appropriate synaptic connections to enable sensory perception and cognitive processing power. Most axon tracts connecting the different brain regions are initiated during embryonic development. The optic pathway and corpus callosum are two axonal pathways which distribute information between the left and right sides of the brain. The corpus callosum comprises axons crossing the midline to interconnect the left and right cerebral hemispheres (Lindwall et al., 2007). The optic pathway comprises retinal ganglion cell (RGC) axons exiting the retina to form the optic nerve before being sorted at the optic chiasm and entering the brain (Erskine and Herrera, 2007). Both have been subject to intensive study so provide good model systems to investigate the function of novel axon guidance molecules.

Heparan sulfate proteoglycans (HSPGs) are secreted and membrane associated proteins attached to unbranched glycosaminoglycan heparan sulfate (HS) molecules (Esko and Selleck, 2002; Kirn-Safran et al., 2009). HSPGs are cofactors in the cellular response to secreted proteins, including Slits and FGFs (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material), involved in the development of axon pathways so HS could play a pivotal coordinating role (Dickson, 2002; Inatani et al., 2003; Johnson et al., 2004; Kantor et al., 2004; Lee and Chien, 2004; Lin, 2004; Hussain et al., 2006; Sánchez-Camacho and Bonvolenta, 2009). HS is critically important for corpus callosum and optic chiasm development as both develop abnormally when HS biosynthesis is blocked in the developing mouse embryonic brain (Inatani et al., 2003). An intriguing feature of HS is its enormous potential for structural diversity, produced by an array of HS modifying enzymes including the heparan sulfotransferases Hs2st and Hs6st1 (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). While it remains conceivable that HS simply provides a nonspecifically permissive environment for the formation of commissural axon tracts, a more interesting possibility is that the rich variation in HS structure generated by the action of HS modifying enzymes is exploited to generate functional diversity in a range of physiological processes including axon navigation (Turnbull et al., 2001; Bülow and Hobert, 2004; Holt and Dickson, 2005; Kreuger et al., 2006).
the telencephalic midline (Erskine et al., 2000; Bagri et al., 2002; Plump et al., 2002; Shu et al., 2003b; Andrews et al., 2006; Thompson et al., 2006; López-Bendito et al., 2007; Plachez et al., 2008). We showed previously that Hs6st1−/− RGCs are less sensitive to Slit2’s chemorepellent activity than wild type RGCs and that defects seen in the optic chiasms of Hs2st−/− and Hs6st1−/− embryos are similar to those seen in Slit/Robo mutants (Pratt et al., 2006). Specifically, Hs6st1−/−, Slit1−/−;Slit2−/−, and Robo2−/− embryos all have increased inter-retinal innervation while Hs2st−/−, Slit1−/−;Slit2−/−, and Robo2−/− embryos all show ectopic wandering of axons along the midline (Plump et al., 2002; Pratt et al., 2006; Plachez et al., 2008).

Here we extend our optic chiasm analysis and show that Hs2st−/− and Hs6st1−/− embryos have distinct cellular and axon guidance defects in the developing corpus callosum. We demonstrate the importance of Hs2st and Hs6st1 in a number of axon navigation events in these systems and identify context-specific roles in Slit/Robo and FGF/FGFR signaling.

Materials and Methods

Animals. The Hs2stLacZ/Hs2st (Hs2st−) mutant allele was obtained by insertion of a LacZ gene trap vector into the Hs2st locus (Bullock et al., 1998) and the Hs6st1LacZ/hplap (Hs6st1−) mutant allele by insertion of a LacZ-ires-hPLAP gene trap vector into intron 1 of the Hs6st1 locus (Leighton et al., 2001; Mitchell et al., 2001). The Hs2st allele and the Hs6st1 allele were maintained on a pigmented C57BL6 background. Embryos used in this study were a mixture of genders and were not sexed. Hs2st animals and embryos in this study were genotyped by multiplex PCR using primers 5′-GGGAAGAAATTCACC-CACAA-3′ and 5′-ATCAATGAATAATTGCCTAGGTC-3′; Slit1, 5′-GGGAAGAAATTCACC-CACAA-3′ and 5′-ATCAATGAATAATTGCCTAGGTC-3′; Slit2, 5′-TCATCTGGAGGAAGGGTGGA-3′; Slit3, 5′-ACCATCTGGTCGAAGGTG-3′; Slit4, 5′-AAGACCTTGCTCTAGGCTAGG-3′; and Slit7, 5′-ACCATCTGGTCGAAGGTG-3′, mutant alleles were main-

Results

Hs2st and Hs6st1 functions in optic chiasm development

Our previous findings suggest the hypothesis that Hs2st and Hs6st1 function in the Robo/Slit signaling pathway in the developing optic chiasm. To investigate this possibility we first re-examined aspects of chiasm development in the two Hst mutants and Slit mutants to allow a more systematic comparison of the phenotypes.

Ectopic retinal innervation in Slit2−/− embryos

Inter-retinal innervation involving RGC axons growing from one eye into the other. Hs6st1−/− embryos have increased inter-retinal innervation compared with Hs2st−/− or wild-type embryos (Pratt et al., 2006). However, we do not have any examples of chiasm development in the two Hst mutants and Slit mutants to allow a more systematic comparison of the phenotypes.
innervation in Slit2−/− and Hs6st1−/− embryos (compare bars 6, 8 in Fig. 1G) suggests a possible shared function for Slit2 and Hs6st1 in this context.

Ectopic RGC axon growth into the Hs2st−/− preoptic area

During normal development RGC axons in the optic nerves converge at the ventral midline posterior to the preoptic area to form the optic chiasm. The combined activities of Slit1 and Slit2 are needed to prevent the optic nerve from invading the preoptic area as Slit1−/−;Slit2−/− and Slit1+/−;Slit2−/− double mutant embryos have an ectopic anterior branch of the optic nerve which crosses the midline at the preoptic area to generate an ectopic chiasm while Slit1−/− and Slit2−/− single mutant embryos do not (Plump et al., 2002) (compare phenotype summaries in Fig. 11D–F). Unilateral Dil labeling of RGC axons in E15.5 Hs2st−/− embryos (Fig. 2B,E,H) revealed an axon bundle branching off the optic nerve and crossing the midline anterior to the main X-shaped optic chiasm (Fig. 2E,H, green arrows) which is absent in wild-type (Fig. 2A,D,G) and Hs6st1−/− (Fig. 2C,F,I) embryos. Neurofilament staining of axons in thin sections shows ectopic axons in the preoptic area in Hs2st−/− (Fig. 2K,N, green arrows) but not wild-type (Fig. 2J,M) or Hs6st1−/− (Fig. 2L,O) embryos. The phenotypes are summarized in Figure 2P–R. Thus, Hs2st activity is required to prevent RGC axons from invading the preoptic area. The similarity between Hs2st−/− single mutant and Slit1−/−;Slit2−/− double mutant preoptic area phenotypes suggests that Hs2st has a possible shared function with both Slit1 and Slit2 in this context.

Genetic interactions between Hst and Slit functions at the optic chiasm

The new findings presented here, in combination with the previously published data, are consistent with a model in which Hs2st and Hs6st1 have unique functions in optic chiasm formation. Our observations that both Hs2st and Slit1 act to prevent ectopic midline wandering (described previously by Pratt et al., 2006)
Slit2<sup>−/−</sup> double mutant embryos. If Hs2st<sub>2</sub>/Slit1 or Hs6st1/Slit2 do indeed act exclusively in the same signaling pathway to regulate a particular process (for example preventing axons navigating into the opposite eye) then the particular aspect of a phenotype (for example increased inter-retinal innervation) in the double mutant will be no more severe than that of either single mutant. Conversely, a more severe phenotype indicates that they act independently of one another. Any additional phenotypes not seen in either single mutant would indicate functional redundancy where loss of either gene can be compensated by the activity of the other, such that the phenotype only appears when both genes are absent. We concentrated on four optic chiasm phenotypes associated with Hst and/or Slit/Robo function (Plump et al., 2002; Pratt et al., 2006; Thompson et al., 2006; Plachez et al., 2008): (1) inter-retinal innervation (Fig. 3, red arrows); (2) ectopic midline wandering (Fig. 3, black arrows); (3) ectopic growth into the preoptic area (Fig. 3, green arrows); and (4) ectopic growth over the ventral telencephalon (Fig. 3, blue arrows). Wild-type (Figs. 1A, G, 3C, F, J, L), Hs2st<sup>−/−</sup>; Slit1<sup>−/−</sup> (Figs. 1E, G, 3A, D, G, J, M), and Hs6st1<sup>−/−</sup>; Slit2<sup>−/−</sup> (Figs. 1F, G, 3B, E, H, K, N) phenotypes are summarized in Figure 3O–Q. Note that while it is straightforward to precisely quantify the magnitude of inter-retinal innervation (by counting retrogradely labeled RGCs) it is not possible to count the numbers of ectopic axons growing down the midline or over the preoptic area or telencephalon. The ectopic tracts were therefore scored blind to genotype on the basis of relative severity judged by careful examination of serial sections from several Dil labeled embryos of each genotype following the approach previously described for Slit mutant chiasm phenotypes (Plump et al., 2002). This semiquantitative analysis is presented in Table 1.

Single mutant Hs2st<sup>−/−</sup> or Slit1<sup>−/−</sup> embryos do not show increased inter-retinal innervation compared with wild-types (Fig. 1B, G) (Pratt et al., 2006, their Fig. 4) and neither do Hs2st<sup>−/−</sup>; Slit1<sup>−/−</sup> double mutant embryos (Fig. 1E, G). While Hs6st1<sup>−/−</sup> and Slit2<sup>−/−</sup> embryos both have an 8–10-fold increase in inter-retinal innervation compared with wild-types (Fig. 1C, D, G) (Pratt et al., 2006, their Fig. 4), Hs6st1<sup>−/−</sup>; Slit2<sup>−/−</sup> embryos have an even greater increase of ~17-fold (Fig. 1F, G) which is not significantly different to that of Slit1<sup>−/−</sup>; Slit2<sup>−/−</sup> embryos (Fig. 1F, compare bars 9, 10) which have previously been reported to have the greatest inter-retinal innervation of all the Slit1; Slit2 single and compound mutants (Plump et al., 2002).

Ectopic midline wandering involves RGC axons escaping from the optic chiasm and growing in a dorsocaudal direction at the thalamic midline (Plump et al., 2002; Pratt et al., 2006) Hs2st<sup>−/−</sup>; Slit1<sup>−/−</sup> double mutant optic axons have ectopic midline wandering similar to the Hs2st<sup>−/−</sup> and Slit1<sup>−/−</sup> single mutants (Table 1; compare midline wandering indicated by black arrows in Hs2st<sup>−/−</sup>; Slit1<sup>−/−</sup> in Fig. 3A, D, J with Hs2st<sup>−/−</sup> in Fig. 2B, H and by Pratt et al., 2006, their Fig. 3). Hs6st1<sup>−/−</sup>; Slit2<sup>−/−</sup> double mutant optic chiasms exhibit a midline wandering phenotype (Fig. 3E, H, K, black arrows) which is interesting as neither Hs6st1<sup>−/−</sup> nor Slit2<sup>−/−</sup> single mutant embryos have a midline wandering phenotype (Plump et al., 2002; Pratt et al., 2006). In
**Figure 3.** *Hs6st1;Slit* double mutant optic chiasm phenotypes at E15.5. **A–H,** Consecutive horizontal sections following unilateral Dil injection into the retina labeling RGC axons at the optic chiasm in E15.5 embryos. Dil images are presented as grayscale negatives for clarity. **A–H,** Wild-type (C, F), *Hs2st1−/−;Slit1−/−* (A, D, G), and *Hs6st1−/−;Slit2−/−* (B, E, H) embryos. *Hs2st1−/−;Slit1−/−* embryos exhibit eecptic RGC axon growth dorsally and caudally (A, D, black arrows) up the midline as well as eecptic growth into the preoptic area (D, green arrow). B, E, H, *Hs6st1−/−;Slit2−/−* embryos exhibit eecptic dorsal and caudal growth up the midline (E, black arrows), eecptic growth into the preoptic area (E, green arrow) and an eecptic tract growing laterally from the optic chiasm (B, blue arrow). I–K, Merges of C and F (I); A, D, and G (J); and B, E, and H (K), with * marking side of Dil application. L—N, Coronal sections through the optic chiasm following unilateral Dil labeling of the retina. N, *Hs6st1−/−;Slit2−/−* embryos an eecptic axon tract grows over the surface of the ventral telencephalon (blue arrow), which is not seen in wild-type (L) or *Hs2st1−/−;Slit1−/−* (M) embryos. O–Q, Diagrams summarizing the phenotypes; green arrows, preoptic area tract; black arrows, midline wandering; red arrows, increased inter-retinal innervation; o, Optic nerve; oc, optic chiasm; ot, optic tract; r, retina; l, lens; poa, preoptic area; M, midline. Scale bars, 500 μm.

fact the *Hs6st1−/−;Slit2−/−* midline wandering phenotype is more severe than seen in *Hs2st1−/−;Slit1−/−* mutants and is comparable to that of *Slit1−/−;Slit2−/−* embryos in which *Slit1* and *Slit2* function are both lost (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (Table 1).

*Hs2st1−/−;Slit1−/−* double mutant embryos have eecptic RGC axon navigation into the preoptic area which is not noticeably more severe than the single mutant *Hs2st1−/−* phenotype (Table 1; compare preoptic area growth indicated by green arrows in *Hs2st1−/−;Slit1−/−* embryos in Fig. 3 D, J with *Hs2st1−/−* embryos in Fig. 2 E, H). *Hs6st1−/−;Slit2−/−* double mutant optic chiasms have growth into the preoptic area which is not seen in either *Hs6st1−/−* or *Slit2−/−* single mutant embryos (compare robust preoptic area growth indicated by green arrows in *Hs6st1−/−*; *Slit2−/−* embryos in Fig. 3 E, K with the lack of phenotype in *Hs6st1−/−* or *Slit2−/−* double mutant embryos). *Hs6st1−/−* embryos in Fig. 2 C, F, I and lack of phenotype described in *Slit2−/−* embryos by Plump et al., 2002, their Fig. 4).

Eccopic growth over the ventral telencephalon involves a bundle of axons branching off the optic tract and growing over the ventral surface of the telencephalon instead of proceeding dorsally over the thalamus. Interestingly, in contrast to the other optic chiasm phenotypes, this tract is far more pronounced in *Slit1−/−*; *Slit2−/−* embryos than in *Slit1−/−;Slit2−/−* embryos indicating that its formation is exquisitely sensitive to *Slit1* dosage and is assisted by retention of some *Slit1* function (Thompson et al., 2006). Eccopic growth over the ventral telencephalon is not seen at all in wild-types (Fig. 3 C, F, I, L) (Thompson et al., 2006), single mutant *Hs2st1−/−* (Fig. 2 B, E, H) or double mutant *Hs2st1−/−;Slit1−/−* embryos (Fig. 3 A, D, G, J, M). While neither *Hs6st1−/−* or *Slit2−/−* single mutant embryos exhibit this phenotype (Fig. 2 G, F, I) (Thompson et al., 2006), an eecptic bundle does branch off the contralateral optic tract and grow over the surface of the telencephalon in *Hs6st1−/−;Slit2−/−* double mutant embryos (compare *Hs6st1−/−; Slit2−/−* in Fig. 3 B, K, N with ectopic tract indicated by blue arrows with *Hs6st1−/−* in Fig. 2 C, F, I and in *Slit1−/−;Slit2−/−* and *Slit1−/−;Slit2−/−* embryos in supplemental Fig. 2, available at www.jneurosci.org as supplemental material). These comparisons are summarized in Table 1 and Figure 11.

What can we deduce from these *Hst/Slit* mutant phenotypes? Loss of *Slit2* function in addition to *Hs6st1* function in the *Hs6st1−/−;Slit2−/−* double mutant experiment results in increased severity of all optic chiasm phenotypes examined compared with the single mutants (Table 1; compare phenotype summaries in Fig. 11 H with C, E). The severity of inter-retinal innervation in *Hs6st1−/−;Slit2−/−* double mutant embryos is greater than in single mutant embryos and preoptic area innervation, midline wandering, and eccopic growth over the ventral telencephalon phenotypes not present in the single mutants at all emerged in the double mutant. Together this shows that *Hs6st1* functions in optic chiasm development independent of *Slit2*. The strongest candidate for the additional factor is *Slit1* based on the close overlap between the *Hs6st1−/−;Slit2−/−* double mutant phenotype and the phenotype of embryos in which *Slit1* and *Slit2* function are simultaneously reduced or abolished [Table 1; compare phenotype summaries in Fig. 11 H with F based on data in Figs. 1, 3 and supplemental Fig. 2 (available at www.jneurosci.org as supplemental material) and by Plump et al. (2002), Thompson et al. (2006), and Plachez et al. (2008)]. The double mutant *Hs2st−/−;Slit1−/−* experiment shows that loss of *Slit1* function in addition to loss of *Hs2st* function does not dras-
navigating the optic chiasm and make abnormal decisions in embryonic embryos might have functional consequences. We analyzed navigation at the optic chiasm (Plachez et al., 2008) we included is not thought to play a role in normal RGC axon navigation at the optic chiasm (Plump et al., 2002; Plachez et al., 2008) we included.

<table>
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<th>Genotype</th>
<th>Preoptic area wandering</th>
<th>Ventral midline wandering</th>
<th>Telencephalic wandering</th>
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<td>Wild-type</td>
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Summary of optic chiasm axon phenotypes in wild-type and Hs2st1, Hs6st1, Slit1, and Slit2 genotypes revealed by DiI injection into the retina at E15.5. For each phenotype each embryo was scored blind to genotype according to the following criteria: −, no phenotype; +, intermediate phenotype; ++, severe phenotype. Examples of the intermediate and severe ventral midline wandering phenotypes are shown in Figs 2E,2H and 3E,4K (black arrows), respectively. The severe preoptic area wandering and telencephalic wandering phenotypes are illustrated in Figs 2E,2H and 3E,4K, respectively.

Table 1. Optic chiasm phenotype of axons labeled by DiI injection into the retina at E15.5. (Incidence/number of embryos scored).

Expression of Robo1/2 and Slit1/2 in Hs2st1/−/− and Hs6st1/−/− retina and chiasm
During normal development of the optic chiasm, RGC axons are kept confined to the highly characteristic X-shaped pathway by the repulsive activities of Slit1 and Slit2, mediated through Robo2 receptors on the navigating axons (Plump et al., 2002; Plachez et al., 2008; Thompson et al., 2009). The similarities between the phenotypes of Hst mutants and Slit/Robo mutants at the optic chiasm could be due to differential sulfation of HSPGs affecting the biochemistry of Slit/Robo signaling or from effects on gene expression, for example, reduced expression of Robo receptors on RGC axons or their Slit ligands at the optic chiasm in Hs2st1/−/− and Hs6st1/−/− embryos. To look for evidence of any such changes in levels or patterns of gene expression in Hs2st1/−/− and Hs6st1/−/− embryos, we examined expression of Robo1 and Robo2 in the retina and Slit1 and Slit2 at the optic chiasm using both in situ hybridization and qRT-PCR. Although Robo1 is not thought to play a role in normal RGC axon navigation at the optic chiasm (Plachez et al., 2008) we included Robo1 in the analysis as changes in its expression level in mutant embryos might have functional consequences. We analyzed E14.5 embryos, the stage at which retinal axons are navigating the optic chiasm and make abnormal decisions in Hs2st1/−/− and Hs6st1/−/− embryos (Pratt et al., 2006).

Using in situ hybridization we found no differences in the distribution of Slit1 (Fig. 4A–C) or Slit2 (Fig. 4E–G) transcripts at the optic chiasm or in the distribution of Robo1 (Fig. 4F–K) or Robo2 (Fig. 4M–O) transcripts in the retinas of either Hs2st1/−/− or Hs6st1/−/− embryos compared with wild types (Fig. 4A, E, I, M) (Erskine et al., 2000) at E14.5.

Using qRT-PCR to assess transcript levels we found that while Slit1 is not altered at the Hs2st1/−/− optic chiasm there was a two-fold reduction in Slit1 at the Hs6st1/−/− optic chiasm (Fig. 4D). Slit2 transcript levels were not significantly affected at the optic chiasm of Hs2st1/−/− or Hs6st1/−/− embryos (Fig. 4H). Neither Robo1 nor Robo2 transcript levels were significantly altered in the retinas of Hs2st1/−/− or Hs6st1/−/− embryos compared with wild-types (Fig. 4L, P). There is no evidence to support the hypothesis that the Hs2st1/−/− or Hs6st1/−/− optic chiasm phenotypes are a consequence of reduced expression of Robo1, Robo2, or Slit2 as we found no significant change in their expression in either genotype. While Slit1 levels are not significantly altered in the Hs2st1/−/− optic chiasm they are reduced at the chiasm of Hs6st1/−/− embryos. This is unlikely to be a cause of the Hs6st1/−/−-inter-retinal innervation phenotype as there is no increase in inter-retinal innervation in Slit1/−/− mutants (Fig. 1B, G) but it could contribute to the phenotype of Hs6st1/−/−;Slit2/−/− embryos where our genetic experiments identified a likely loss of Slit1 function.

Hs2st and Hs6st1 functions in corpus callosum development
To investigate whether Hs2st and Hs6st1 are required for Slit/Robo-mediated axon guidance at sites other than the optic chiasm, we examined formation of the corpus callosum in the Hst mutants. The corpus callosum is a commissural tract containing axons that form reciprocal connections between neurons in layers 2,3 and 5 of the cerebral hemispheres (Silver et al., 1982; Katz et al., 1983; Silver and Ogawa, 1983). Cerebral cortical axons navigate mediially toward the midline through the intermediate zone with the first callosal axons crossing the midline at E16.5 and more axons crossing during subsequent embryonic development and for the first few postnatal days. The midline crossing point is a structure formed by the fusion of the cerebral hemispheres just above the septum which, by E18.5, is surrounded by populations of neurons and glia in the indusium griseum (IG), glial wedge (GW), midline zipper (MZ), and subcallosal sling (SCS) (Lindwall et al., 2007; Paul et al., 2007).

Differential requirement for Hs2st and Hs6st1 in midline axon navigation at the corpus callosum
Using carboxycyanine dye tract tracing to assess formation of the corpus callosum in Hs2st1/−/− and Hs6st1/−/− embryos at E18.5, we found distinct defects in each mutant. At this stage in wild-type embryos cortical axons have crossed the telencephalic midline above the septum to form a smooth U-shaped tract (Fig. 5A, D, G) with axon crossing observed in 5/5 embryos examined. In Hs2st1/−/− embryos (Fig. 5B, E, H), axons frequently failed to cross the telencephalic midline at all (4/8 embryos) and formed abnormal or subcallosal knob-like structures resembling Probst bundles on either side of the midline (5/8 embryos). In some cases we were unable to detect any gross axon guidance defects (3/8 embryos), indicating that this phenotype is partially penetrant. In all of the Hs6st1/−/− embryos, callosal axons failed to cross the midline and formed abnormal knob-like structures on either side of the midline (Fig. 5C, F, I) (6/6 embryos). We also frequently saw ectopic growth of axons ventrally into the septum (4/6 embryos) (Fig. 5C, F, G) which was not apparent in wild-type or Hs2st1/−/− embryos. These corpus callosum axon navigation phenotypes are summarized in Figure 5J–L. In conclusion, Hs2st and Hs6st1 are each critical for the normal projection of axons across the corpus callosum and differences in their loss of function phenotypes

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suggest that each has a different function in the normal development of the corpus callosum.

Development of telencephalic midline neuronal structures in Hs2st\(^{-/-}\) and Hs6st1\(^{-/-}\) embryos

Neurons at the telencephalic midline function in guiding callosal projections by contributing to the SCS and can be visualized by staining for the transcription factor Tbr1 (Niquille et al., 2009). In addition to marking postmitotic neurons in the SCS, Tbr1 also labels postmitotic neurons in the IG (Piper et al., 2009).

At E16.5 we were unable to detect any differences in Tbr1 staining between wild-type (Fig. 6A), Hs2st\(^{-/-}\) (Fig. 6B), and Hs6st1\(^{-/-}\) embryos in the region where the corpus callosum will subsequently form, indicating that this region is not grossly mis-patterned in the mutants at this stage.

In wild-type embryos by E18.5 Tbr1-expressing cells form a U-shaped sling ventral to the corpus callosum axon bundle (Fig. 6D, SCS) and mark the IG region dorsal to the corpus callosum. In Hs2st\(^{-/-}\) embryos the IG staining is still apparent but the Tbr1-expressing cells are absent from the telencephalic midline (Fig. 6E, white arrow). In Hs6st1\(^{-/-}\) embryos the IG staining is also apparent but the Tbr1-expressing cells are clustered around the knotted axon bundles flanking the telencephalic midline (Fig. 6F, white arrow). Thus, Tbr1-expressing cells are unable to form a normal subcallosal sling in the absence of Hs2st or Hs6st1 function but instead remain associated with the noncrossing callosal axon bundles. The presence of Tbr1-expressing neurons marking the IG in wild-type, Hs2st\(^{-/-}\), and Hs6st1\(^{-/-}\) embryos (Fig. 6D–F, dashed box) indicates that the IG develops in its normal position in both mutants.
Differential requirement for Hs2st and Hs6st1 during the development of glial structures required for corpus callosum formation

We next analyzed the formation of the glial structures that are critical to corpus callosum formation using a glial cytoskeletal marker, GFAP, from E16.5 to E18.5.

In wild-type embryos at E16.5 GFAP staining is detected in large numbers of glial processes which extend from the ventricular surface of the corticoseptal boundary toward the midline to form the GW (Fig. 6G, yellow arrow). At E16.5 the GW glial processes are visible in Hs2st−/− embryos (Fig. 5H, yellow arrow) although the staining is fainter and less extensive suggesting a reduction in the number of GFAP-expressing glia. In Hs6st1−/− embryos at E16.5 only a very few GFAP-expressing glia are seen at the GW (Fig. 6I, yellow arrow). To quantify the GW glial phenotype we measured the distance between the dorsal- and ventral-most GFAP stained glial processes in the left and right hand side corticoseptal ventricular zone in consecutive coronal sections [2–3 × 100 µm sections measured for each E16.5 embryo] spanning the full rostro-caudal extent of the corticoseptal boundary (for illustration of GW measurement, see Fig. 6M). Comparison of this measurement between genotypes at E16.5 revealed a 35% reduction in Hs2st−/− embryos and a 69% reduction in Hs6st1−/− embryos compared with wild-type embryos (Fig. 6N).

The development of the GW glia is therefore defective in both mutants with the most severe defect in Hs6st1−/− embryos.

By E17.5 in wild-type embryos (Fig. 6J) GFAP staining has expanded in the GW, and GFAP-expressing glia are found in the IG (Fig. 6J, dotted box) dorsal to the corpus callosum at the midline. By E17.5 we were unable to identify differences in GFAP staining at the GW in wild-type (Fig. 6J, yellow arrow), Hs2st−/− (Fig. 6K, yellow arrow), and Hs6st1−/− (Fig. 6L, yellow arrow) embryos indicating that the GW phenotype at E16.5 represents a developmental delay rather than a failure of glial differentiation at the GW [a comparison of GW measurements at E17.5 (taken from 3–4 × 100 µm sections from each embryo) is shown in Fig. 6O]. In E17.5 Hs2st−/− embryos, glia are present in the IG (Fig. 6K, dotted box) but some glia occupy ectopic positions at the telencephalic midline (Fig. 6K, blue arrow). In E17.5 Hs6st1−/− embryos, glia are extremely disorganized at the telencephalic midline where they form a continuous strip (Fig. 6L, blue arrow) and ectopic streams of glia coincide with knotted axon bundles at the telencephalic midline and extend laterally toward the GW (Fig. 6L, black arrow) and are absent from the IG (Fig. 6L, dotted box). E17.5 Hs6st1−/− vibratome sections frequently exhibited separation of the cerebral hemispheres (Fig. 6L, *) indicating a mechanical defect in inter-hemispheric connection at this stage. At E18.5 large numbers of glia are present in the path of callosal axons in both Hs2st−/− (Fig. 6R) and Hs6st1−/− (Fig. 6S) embryos in contrast to their normal positions in the wild-type (Fig. 6P,Q) and are absent from the IG (Fig. 6S, dotted box) in Hs6st1−/− embryos indicating that the absence of glia from the Hs6st1−/− IG is delayed by >1 d. The very low survival rate of Hs6st1−/− embryos after birth prevented us from assessing whether glia ever subsequently populate the IG. The neuronal structures of the IG, as defined by Tbr1 staining, are comparable in all genotypes (Fig. 6D–F, dashed box) so the specific inability of GFAP-expressing glia to populate the IG in Hs6st1−/− (but not Hs2st−/−) embryos indicates a specific Hs6st1−/− glial cell phenotype. In conclusion we find that Hs2st and Hs6st1 are each critical, in distinct ways, for the generation of telencephalic glial structures required for
Many ectopic glia are seen along the midline (blue arrow) and medial to the GW (black arrow). The cerebral hemispheres have corpus callosum axons (blue arrow).

**Figure 6.** Development of telencephalic midline neuronal and glial structures critical for corpus callosum development in Hs2st−/− and Hs6st1−/− embryos. A–F, Tbr1 (green) is a marker for neurons that populate the IG and the SCS. A–C, E16.5. A, In wild-type embryos, Tbr1-expressing cells are located in the cortex and into the IG region (dotted box). This pattern is not altered in Hs2st−/− (B) or Hs6st1−/− (C) embryos. D–F, E18.5. D. In wild-type embryos Tbr1-expressing cells form a continuous sling at the ventral border of the corpus callosum called the SCS. Densely packed cells expressing high levels of Tbr1 mark the IG region (enclosed by dotted box) with less dense labeling in the cerebral cortex. E, Although Hs2st−/− embryos have cortical and IG (dotted box) staining similar to wild-type embryos, the SCS pattern is different with a gap (white arrow) devoid of Tbr1-positive cells at the midline. F, In Hs6st1−/− embryos densely packed Tbr1-expressing cells mark the IG (dotted box) but the Tbr1-expressing cells do not span the midline and there is a gap in the sling (white arrow). G–I, P–R, Immunohistochemistry for GFAP labels glia (P–R) are double labeled with the axonal marker L1 (red). G–J, E16.5. G, In wild types, glia are arranged in the GW in the ventricular zone of the corticoseptal boundary (yellow arrow). H, In Hs2st−/− embryos the GFAP pattern is comparable to wild types at the GW (yellow arrow). I, Hs6st1−/− embryos have a more severe phenotype with very few glia found in the GW at this stage (yellow arrow, glial fascicle). J–L, By E17.5 there are no longer gross differences in the GW (yellow arrows) but glial organization at the midline differs dramatically between genotypes. J, In wild-type embryos, glia have populated the IG above the corpus callosum. K, In Hs2st−/− embryos, glia are present in the IG (dotted box) and there are a few ectopic glia in the path of corpus callosum axons (blue arrow). L, In Hs6st1−/− embryos there are no glia populating the region of the IG (dotted box) and many ectopic glia are seen along the midline (blue arrow) and medial to the GW (black arrow). The cerebral hemispheres have become separated with the gap marked by an asterisk. M–O, Quantification of GFAP staining at the GW in wild-type, Hs2st−/−, and Hs6st1−/− embryos. M, Diagram illustrating the measurement of the length of the GW taken from the dorsal-most to the ventral-most GFAP stained glial process as described in Results. N, At E16.5 there are significant differences between all genotypes and the GW is by far the smallest in Hs6st1−/− embryos. O, At E17.5, there is no significant difference between genotypes. Error bars are ±SEM with the number of embryos analyzed shown below each bar. Numbers above each bar indicate Student’s t-test p values for comparison to wild type or bracketed for comparison between mutants. Significant differences (p < 0.05) in bold. P–S, At E18.5 glia occupy the IG in wild-type (P, Q) and Hs2st−/− (R) embryos but absent from the IG in Hs6st1−/− (S) embryos (dashed boxes in Q–S enclose IG). More glia are seen at the midline in Hs2st−/− embryos than in wild types (R, blue arrow) and in Hs6st1−/− embryos large numbers of ectopic glia are found both at the midline (S, blue arrow) and forming ectopic strands intermingled with the axons (S, white arrow), s, Septum; vz, ventricular zone; ctx, cerebral cortex. All sections coronal with dorsal at the top. Scale bars: A–L, P, 200 μm; Q–S, 100 μm.
ers (presumptive layers 1 and 6) (Fig. 7G) while Hs6st1 is restricted to intermediate layers (presumptive layers 3–5) with a prominent strip of nonexpressing cells separating a thin, deep expressing population from a broader, more superficial expressing population (Fig. 7H). At the telencephalic midline, Hs2st and Hs6st1 are both expressed in the IG region (Fig. 7J, L, red arrows) dorsal to callosal axons with Hs2st expression prominent at the GW. Ventral to the corpus callosum, Hs2st is expressed in the region of the subcallosal sling (Fig. 7I, yellow arrows) and Hs6st1 is expressed in the septum (Fig. 7L).

Thus, Hs2st and Hs6st1 are each expressed in distinct sets of cells in regions known to be important for corpus callosum formation. Hs2st is not expressed at high levels in cortical cells projecting axons to the corpus callosum but is expressed in the GW, IG, and SCS, consistent with a possible role in the generation of the midline environment required for callosal axon crossing. Hs6st1 is expressed in cortical layers which project axons to the corpus callosum as well as in the IG and GW consistent with a possible role both in navigating axons themselves and in the generation of the midline environment. To further address a possible cell autonomous role for Hs6st1 in callosal axon navigation we took advantage of the human placental alkaline phosphatase (hplap) cassette included in the Hs6st1 gene trap reporter allele (Mitchell et al., 2001) which marks axons of expressing cells. At E18.5 hplap reporter expression is very weak in the corpus callosum axon bundle indicating low (if any) Hs6st1 expression in cortical cells projecting axons to the corpus callosum (Fig. 7I, compare with strong hplap staining of the anterior commissure in the same section in I′, which provides a positive control). The low Hs6st1 expression in callosal projection neuron argues against a cell autonomous role for Hs6st1 in cortical axon navigation in the corpus callosum.

Roles for Hs2st and Hs6st1 in midline cell positioning

The positioning of specific populations of cells at the midline is critical for normal development of the corpus callosum (Lindwall et al., 2007; Paul et al., 2007). One possible function for Hs2st or Hs6st1 might be to regulate neuronal or glial positioning cell autonomously so we next asked whether the cells marked by high expression of Hs2st or Hs6st1 are positioned normally in each Hst mutant. We examined the locations of LacZ-expressing cells in Hs2st−/+ and Hs6st1−/+ embryos. Cells marked by Hs2st-LacZ gene expression (but unable to make functional Hs2st protein) in Hs2st−/+ embryos occupy the same positions as their heterozygous counterparts in the GW at E16.5 and E18.5 (Fig. 7D, K) with strong staining of the IG at E18.5 (Fig. 7K, red arrows). Strongly stained cells are apparent at the midline in the normal path of callosal axons at E18.5, coinciding with ectopic glia in Hs2st−/+ embryos (Fig. 6K). Further, Hs2st-LacZ is no longer expressed continuously along the midline ventral to callosal axons (Fig. 7K) reminiscent of the noncontinuous SCS labeling by Tbr1 in Hs2st−/+ embryos (Fig. 6E, white arrows). At E16.5 there is little difference in the distribution of Hs6st1-LacZ-expressing cells in Hs6st1−/+ embryos compared with Hs6st1−/+ embryos (Fig. 7, compare P with E). However, by E18.5 there are clear differences with Hs6st1-LacZ-expressing cells in Hs6st1−/+ embryos failing to populate the IG and instead occupying ectopic locations at the corticoseptal boundary and intermingling with callosal axons (Fig. 7M, dashed red arrow) in clumps reminiscent of the ectopic clusters of glia seen in Hs6st1−/+ embryos (Fig. 6S, white arrow). In conclusion cells marked by Hs2st and Hs6st1 gene expression end up in ectopic locations in their respective loss of function mutants consistent with a cell autonomous role for Hs2st and Hs6st1 in normal cell positioning at the midline.

Figure 7. Expression of Hs2st and Hs6st1 in the formation of the corpus callosum. Hs2st and Hs6st1 expression analysis using LacZ and hplap gene-trap reporter transgenes. A–M, Hs2st+/− (A, C, G, J), Hs2st−/− (D, K), Hs6st1+/− (B, E, H, I, F, L), and Hs6st1−/− (F, M) embryos at E16.5 (A–F) and E18.5 (G–M). A–H, J–M, LacZ histochemistry (blue). I, I′, Hplap histochemistry (purple). At E16.5 both Hs2st (A, C) and Hs6st1 (B, E) are expressed widely in the telencephalon including the cerebral cortex, ventral telencephalon, and septum with variations in staining intensity across these structures. D) Hs2st−/+ embryos show similar GW staining to (C) Hs2st+/− embryos. F, Hs6st1+/− embryos have similar patterns of expression to (E) Hs6st1−/− embryos. G–M, At E18.5 LacZ expression in Hs2st−/− (G) and Hs6st1−/− (H) embryos are in distinct laminar patterns in the cerebral cortex. G, H, Red boxes, Regions shown at higher magnification in J and L. I, Weak hplap staining of corpus callosum axons in coronal section from Hs6st1−/− embryo. I′, Strong hplap staining of the anterior commissure crossing the midline at a more ventral location in the same section. J, Hs2st+/− LacZ expression is high in the glial wedge and in the IG (red arrow) and the SCS (yellow arrow). K, The IG shows high LacZ expression in Hs2st−/−, however unlike the Hs2st+−/+ (J), there is high LacZ expression at the midline below the IG. L, Hs6st1+/− LacZ is expressed strongly dorsal to the corpus callosum in the IG (red arrow). M, Hs6st1−/− embryos have ectopically located LacZ cells intermingled with the corpus callosum axon bundles (dashed red arrow) and no apparent LacZ expression in the IG. G, H, Red boxes, regions shown at higher magnification. C, F, I, M, 500 μm. D–M, 250 μm.
Table 2. Corpus callosum phenotype of axons labeled by DiI injection into the cerebral cortex at E18.5 (incidence/number of embryos scored)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Severity of midline crossing defect</th>
<th>Axon whorls flanking midline</th>
<th>Septal axons ventral to the corpus callosum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>− (5/5)</td>
<td>− (5/5)</td>
<td>− (5/5)</td>
</tr>
<tr>
<td>Hs2st&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>− (4/8), + + (4/8)</td>
<td>+ + (6/6)</td>
<td>+ + (6/6), − (2/6)</td>
</tr>
<tr>
<td>Hs6st1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+ + (6/6), − (3/8), + (4/4)</td>
<td>+ + (6/6)</td>
<td>+ + (6/6)</td>
</tr>
<tr>
<td>Slit2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+ (3/3)</td>
<td>+ + (6/6)</td>
<td>+ + (3/3)</td>
</tr>
<tr>
<td>Slit2&lt;sup&gt;−/−&lt;/sup&gt;;Hs6st1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+ + (3/3), + + (1/4)</td>
<td>+ + (6/6)</td>
<td>+ + (4/4)</td>
</tr>
<tr>
<td>Slit2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>− (3/3)</td>
<td>− (3/3)</td>
<td>− (3/3)</td>
</tr>
<tr>
<td>Slit2&lt;sup&gt;−/−&lt;/sup&gt;;Slit1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>+ (2/2)</td>
<td>+ + (2/2)</td>
<td>+ + (2/2)</td>
</tr>
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</table>

Summary of corpus callosum axon phenotypes in wild-type and Hs2st, Hs6st1, Slit1, and Slit2 complete loss of function genotypes revealed by DiI injection into the cerebral cortex at E18.5. Severity of midline crossing defect: −, large numbers of callical axons crossing the midline indistinguishable from wild types; +, diminished crossing (Fig 4A–G); + +, absolutely no crossing (Fig 5C,F). Probst-like axon whorls flanking the midline were scored as follows: −, absent; +, +, present (Fig 5C,F). Septal axon phenotypes were as follows: −, no phenotype compared with wild type; +, intermediate phenotype (Fig 5C,F); + +, severe phenotype (Fig 9–CE).

Genetic interactions between Hs6st1 and Slit2 functions at the corpus callosum

Slit2 is the key Slit for corpus callosum development

The Slit2<sup>−/−</sup> corpus callosum phenotype comprises Probst-like bundles, prolific ectopic ventrally deflected septal axons, and a reduction in the numbers of axons able to cross the midline (Bagri et al., 2002). Our analysis of the optic chiasm indicated roles for Hs6st1 and Hs2st in Slit1 and Slit2 function and we wondered whether the same was the case at the corpus callosum. The role of Slit1 in corpus callosum development has not yet been reported. We find that Slit1<sup>−/−</sup> embryos (3/3) have no discernible corpus callosum axon phenotype (supplemental Fig. 3A, available at www.jneurosci.org as supplemental material) and we were unable to detect any disorganization of the midline glia (supplemental Fig. 3B, available at www.jneurosci.org as supplemental material). The Slit1<sup>−/−</sup>;Slit2<sup>−/−</sup> callosal axon phenotype (supplemental Fig. 3G–I, available at www.jneurosci.org as supplemental material) (2/2) is indistinguishable from the Slit2<sup>−/−</sup> phenotype (Table 2) (supplemental Fig. 3C,D, available at www.jneurosci.org as supplemental material). A relatively mild midline gli disorganization phenotype has previously been reported for Robo1<sup>−/−</sup> embryos at E18.5 (Andres et al., 2006) and this is closely phenocopied by Slit2<sup>−/−</sup> embryos (arrow in supplemental Fig. 3F, available at www.jneurosci.org as supplemental material). Together these findings indicate that Slit2 is responsible for Slit/Robo function in the development of the corpus callosum and that Slit1 has a minor, if any, role in the development of this tract.

Genetic interaction between Hs6st1 and Slit2 in corpus callosum development

Hs6st1<sup>−/−</sup> and Slit2<sup>−/−</sup> embryos share two corpus callosum phenotypes namely the formation of Probst-like axon whorls flanking the midline and axons wandering ventral to the corpus callosum in the septum. The relative severity of the phenotypes differs between Hs6st1<sup>−/−</sup> and Slit2<sup>−/−</sup> genotypes with Hs6st1<sup>−/−</sup> embryos presenting a more severe midline crossing phenotype and Slit2<sup>−/−</sup> embryos a more severe septal axon wandering phenotype (Table 2). This observation is consistent with Hs6st1 normally functioning to facilitate the corpus callosum axon guidance function of Slit2. We hypothesized that if Hs6st1 and Slit2 act in synergy then simultaneous reduction of gene dosage in compound heterozygous Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> embryos would provoke more severe corpus callosum phenotypes than occur in Slit2<sup>−/−</sup>, Hs6st1<sup>−/−</sup>, or wild-type embryos. DiI/DiA tract tracing from the cerebral cortex revealed that Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> (3/3) embryos formed a U-shaped corpus callosum (Fig. 8D) which was indistinguishable from wild-type (4/4) (Fig. 8A), Hs6st1<sup>−/−</sup> (4/4), and Slit2<sup>−/−</sup> (3/3) (not shown) embryos and we did not detect Probst-like axon whorls flanking the midline in any of these genotypes (Table 2). In contrast there were more DiI labeled axons ventral to the corpus callosum in Hs6st1<sup>−/−</sup>; Slit2<sup>−/−</sup> embryos compared with wild types (Fig. 8, compare F, with C, arrows point to septal axons in the area indicated by dashed boxes in A,D). To analyze this systematically we scored serial coronal sections covering the rostral-caudal extent (section 1 most caudal to section 7 most rostral) of the corpus
Slit2 blocks callosal axon midline crossing in Hs6st1<sup>−/−</sup> embryos

Examination of corpus callosum development in embryos simultaneously lacking both Hs6st1 and Slit2 function reveals an intriguingly complex, and nonepistatic, relationship between these two genes in generating the Hs6st1<sup>−/−</sup> mutant phenotype. In Hs6st1<sup>−/−</sup> embryos the failure of callosal axons to cross the midline is accompanied by large numbers of ectopic glia lining up along the midline (Fig. 6L,S). During normal development Slit2, produced by glia in the GW and IG, guides callosal axons by repulsive interactions (Bagri et al., 2002; Shu et al., 2003b) so we hypothesized that a contributory factor to failed midline crossing in Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos is Slit2-mediated repulsion from the midline caused by the ectopically located glia. This hypothesis predicts that the additional removal of Slit2 function in an Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryo would attenuate the repulsive force exerted by the mutant midline environment on callosal axons and rescue midline crossing. Dil injection into the cerebral cortex of E18.5 Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> embryos labels abnormally large numbers of axons growing in the septum (Fig. 9A–C) and Probst-like axon whorls flanking the midline (Fig. 9D,E) indicating that this genotype has a major callosal defect. More interestingly, we saw callosal axons crossing the midline in 3/4 embryos examined although crossing was obviously not rescued to wild-type levels (Fig. 9F) so we hypothesized that a contributory factor to failed midline crossing in Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos is Slit2-mediated repulsion from the midline caused by the ectopically located glia. This hypothesis predicts that the additional removal of Slit2 function in an Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryo would attenuate the repulsive force exerted by the mutant midline environment on callosal axons and rescue midline crossing. Dil injection into the cerebral cortex of E18.5 Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> embryos labels abnormally large numbers of axons growing in the septum (Fig. 9A–C) and Probst-like axon whorls flanking the midline (Fig. 9D,E) indicating that this genotype has a major callosal defect. More interestingly, we saw callosal axons crossing the midline in 3/4 embryos examined although crossing was obviously not rescued to wild-type levels (Fig. 9B–D; Table 2). This rescue is consistent with the hypothesis that Slit2 function blocks callosal axons from crossing the midline in Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos. GFAP/L1 staining revealed that large numbers of ectopic glia are present at the midline of Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> embryos (Fig. 9E) but, in contrast to the Hs6st1<sup>−/−</sup> phenotype, the axons have grown through them consistent with the idea that the
loss of Slit2 function makes them less repulsive. Critically this phenotype shows that Hs6st1 must have Slit independent functions in the developing corpus callosum.

In Hs6st1<sup>−/−</sup> and Slit2<sup>−/−</sup> embryos we observed increased numbers of axons in the septum ventral to the corpus callosum with a more severe phenotype in Slit2<sup>−/−</sup> embryos (Fig. 5C,F,I; Table 2) (supplemental Fig. 3CE, available at www.jneurosci.org as supplemental material). The amount of septal axon growth in the Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos was comparable to the Slit2<sup>−/−</sup> phenotype (Table 2; compare Fig. 9A,E to supplemental Fig. 3CE, available at www.jneurosci.org as supplemental material). Therefore, in contrast to midline crossing, the septal axon phenotype was not rescued in Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos and, as we did not detect any obvious enhancement of this phenotype compared with, Slit2<sup>−/−</sup> embryos, this is consistent with a more straightforward epistatic relationship between Hs6st1 and Slit2 reminiscent of our findings at the optic chiasm (Pratt et al., 2006, present study).

FGF signaling is reduced in the corticoseptal region in Hs6st1<sup>−/−</sup> embryos

At the optic chiasm and in the optic tracts the spectrum of RGC axon navigation properties found in Hs2st<sup>−/−</sup>, Hs6st1<sup>−/−</sup>, Hs2st<sup>+/−</sup>; Slit1<sup>−/−</sup>, and Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos closely overlap, suggesting that these Hsts and Slits function in the same signaling pathways (Table 1; see Fig. 11). Hs6st1<sup>−/−</sup> mutants have more severe defects in corpus callosum axon midline crossing and the production of glial structures than do Slit1<sup>−/−</sup>, Slit2<sup>−/−</sup>, Robo2<sup>−/−</sup> or Slit1<sup>−/−</sup>/Slit2<sup>−/−</sup> embryos (Bagri et al., 2002; Richards, 2002; Andrews et al., 2006; Lopez-Bendito et al., 2007) (Table 2; see Fig. 11, compare K with L), indicating that Hs6st1 likely acts in one or more additional pathways to Slit/Robo. Further support for this idea comes from the analysis of Hs6st1<sup>−/−</sup>; Slit2<sup>−/−</sup> embryos which provide clear evidence for nonepistatic relations between Slit2 and Hs6st1. The absence of glia in the IG and the ectopically located glia that we find in Hs6st1<sup>−/−</sup> embryos, neither of which is seen in Robo/Slit mutants (Bagri et al., 2002; Andrews et al., 2006), resemble the phenotype reported in conditional FGFR1<sup>−/−</sup> mutant embryos, which have reduced levels of FGF signaling (Smith et al., 2006; Tole et al., 2006; Moldrich et al., 2010) (compare genotype summaries in Fig. 11 K and M, N). The normal differentiation and migration of glia which form the GW and IG starts at E14.5 and requires FGF signaling (Smith et al., 2006; Tole et al., 2006). To determine whether reduced FGF signaling could contribute to the Hs6st1<sup>−/−</sup>/Slit2<sup>−/−</sup> callosal phenotype we examined the expression of Sprouty2, a transcriptional target of FGF signaling, at the telencephalic midline. In situ hybridization did not reveal any clear differences in the expression pattern of Sprouty2 between E14.5 wild-type and Hs6st1<sup>−/−</sup> embryos, both of which showed high expression at the corticoseptal boundary where the IG glia are born before translocating to the IG (Fig. 10A,B, red arrow). We quantified the levels of FGF signaling by measuring Sprouty2 mRNA levels in tissue dissected from E14.5 telencephalon comprising the medial cortex, corticoseptal boundary and septum but excluding the ganglionic eminences and more lateral cortex. We found a significant 25% reduction in Sprouty2 levels in Hs6st1<sup>−/−</sup> embryos compared with wild-types (Fig. 10C) confirming that Hs6st1 is required to achieve normal FGF signaling levels. These findings are consistent with a model in which Hs6st1 function is required to attain the FGF signaling levels required for proper glial cell differentiation.

**Discussion**

**The optic chiasm**

Similarities between Hst and Slit/Robo optic chiasm phenotypes (present study and (Plump et al., 2002; Pratt et al., 2006; Thompson et al., 2006; Plachez et al., 2008) (summarized in Fig. 11A–F; Table 1) suggest that Hs2st and Hs6st1 function in the same pathways as Slit1, Slit2, and Robo2. Here we used genetic analysis to elucidate the detail of the Hst/Slit/Robo interactions at the optic chiasm. The optic chiasm phenotypes of embryos with single or compound Hs2st<sup>−/−</sup>, Hs6st1<sup>−/−</sup>, Slit1<sup>−/−</sup>, or Slit2<sup>−/−</sup> loss of function genotypes are best explained by a model in which Hs2st and Hs6st1 are both capable of mediating the function of Slit1 and/or Slit2 depending on the context. Our data show that the function of Hs2st and Hs6st1 at the optic chiasm can mainly be accounted for by a role in mediating Slit1 and Slit2 function. Mechanistically, Hsts might exert their influence on Robo/Slit signaling biochemically by regulating the formation of the Robo/Slit/HS signaling complex (Hussain et al., 2006), the distribution of Slit proteins (Johnson et al., 2004), or gene expression. HSPGs modulate the activity of morphogens, including Wnt, Shh and FGF, which control gene expression (Perrimon and Bernfield, 2000; Selleck, 2000; Lin, 2004). We found decreased levels of Slit1 transcripts at the optic chiasm of Hs6st1<sup>−/−</sup> embryos. Reduced Slit1 is unlikely to account for the inter-retinal guidance phenotype in Hs6st1<sup>−/−</sup> embryos, as even complete loss of Slit1 function alone does not result in increased inter-retinal innervation. Reduced Slit1 could contribute to the Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> optic chiasm phenotype (present study) and explain the phenocopy between Hs6st1<sup>−/−</sup>;Slit2<sup>−/−</sup> and Slit1<sup>−/−</sup>;Slit2<sup>−/−</sup> embryos (Table 1, Fig. 11) (Plump et al., 2002; Thompson et al., 2006). Hs6st1 might regulate Slit1 expression via the Slit/Robo or FGF/FGFR pathways as (1) Hsts regulate Slit/Robo signaling (present study) and Slit/Robo signaling regulates gene expression by inducing phosphorylation and nuclear translocation of β-catenin (Rhee et al., 2002, 2007) and (2) Hsts regulates FGF...
signaling (Grobe et al., 2005) (present study) and in *Xenopus* FGF signaling up-regulates Slit1 (but not Slit2) expression in the diencephalon (Atkinson-Leadbeater et al., 2010).

Although interactions between Hs6st1 and Slit1/Slit2 appear to dominate at the optic chiasm, interactions with additional factors cannot be ruled out. One candidate is the secreted morphogen Shh which can guide RGC axons and whose signaling activity depends on HSPGs (Trousse et al., 2001; Grobe et al., 2005; Dierker et al., 2009a,b). Disrupting Shh or its receptor BOC produces increased ectopic RGC axon navigation into the contralateral optic nerve reminiscent of the Hs6st1−/− phenotype (Sánchez-Camacho and Bovolenta, 2008; Fabre et al., 2010).

The corpus callosum

Corpus callosum development is a complex event requiring the precise coordination of tissue fusion, neuron and glial cell production, cell migration, and axon guidance molecule expression (Serafini et al., 1996; Bagri et al., 2002; Shu et al., 2003a,b; Andrews et al., 2006; Keeble et al., 2006; Smith et al., 2006; Tole et al., 2006; Lindwall et al., 2007; López-Bendito et al., 2007; Paul et al., 2007; Islam et al., 2009; Niquille et al., 2009). Embryos lacking the HS biosynthetic enzyme Ext1 in the developing CNS exhibit severe morphological distortions at the telencephalic midline and axons fail to connect the cerebral hemispheres illustrating the vital importance of HS in this developing system (Inatani et al., 2003). HS modification is also critical as embryos lacking the heparan sulfotransferase Ndst1 have major midline morphological defects and no axon crossing in the corpus callosum and embryos lacking the Sulf heparan endosulphatases have corpus callosum dysgenesis (Grobe et al., 2005; Kalus et al., 2009).

Our data fit a model in which Hs2st and Hs6st1 have context specific functions in generating the midline cellular environment needed for callosal axon navigation. First, Hs2st and Hs6st1 are regionally expressed around the telencephalic midline with high expression at positions coinciding with key sites of gliogenesis and differentiation in the developing GW and IG. Second, cells marked by Hs2st and Hs6st1 expression are mislocated in Hs2st−/− and Hs6st1−/− embryos consistent with a cell autonomous role in cell positioning. Third, the midline glial cell populations, whose normal function is to guide callosal axons by secreting guidance cues including Slit2 (Shu et al., 2003b; Lindwall et al., 2007; Paul et al., 2007) develop abnormally in Hs2st−/− and Hs6st1−/− embryos with a more severe glial phenotype in Hs6st1−/− embryos at the GW and IG. The IG is populated by glia which originate in the GW (Smith et al., 2006) so the delay in GW glial maturation may well contribute to the

Figure 11. Summary and comparison of optic chiasm and corpus callosum phenotypes. A–H, The optic chiasm. Diagrams show RGC axons navigating through the optic chiasm in the genotypes indicated at E15.5. Phenotypes: green arrow, ectopic growth though the poa; black arrow, midline wandering; red arrow, growth into the opposite eye; blue arrow, ectopic tract growing over the ventral telencephalon. A, Wild-type. B–E, Hst and Slit single mutant phenotypes (data in Figs. 1, 3) (Plump et al., 2002; Pratt et al., 2006). F, Slit1−/−;Slit2−/− diagram based on data from Plump et al. (2002), Thompson et al. (2006), and Plachez et al. (2008). G, H, Hst and Slit double mutant phenotypes (data in Figs. 2, 3). I–N, The corpus callosum. Diagrams show axons navigating through the corpus callosum and the distribution of GFAP-expressing glia (green ovals) in the GW, IG, and MZ in the genotypes indicated at E18.5. Phenotypes: gray arrow, ectopic growth into the septum; P, Probst-like knotted axons; *, severe midline glial defect; purple arrow, callosal axons crossing the midline. I, Wild-type. J, K, Hst single mutants (data in Figs. 5, 6). L–M, Slit/Robo and FGFR mutants. L, Slit2−/−; Robo1−/−; Robo1−/−, and Robo1−/−;Robo2−/− (Bagri et al., 2002; Andrews et al., 2006; López-Bendito et al., 2007). Foxg1Cre;FGFR1Fl/Fl conditional knockout of FGFR1 throughout the developing telencephalon using Foxg1Cre in which midline glia are absent (Tole et al., 2006) (M). O, Hs6st1−/−;Slit2−/− embryos in which callosal axons are able to cross the midline, in contrast to the Hs6st1−/− phenotype (compare with K) where axons do not cross, and despite a major midline glial disorganization phenotype. r, Retina; l, lens; on, optic nerve; oc, optic chiasm; ot, optic tract; poa, preoptic area; ctx, cerebral cortex; vtel, ventral telencephalon; s, septum; P, Probst-like knotted axons.
Hs6st1 with Slit/Robo differs (Bagri et al., 2002; Andrews et al., 2006; López-Bendito et al., 2007) (Table 2; Fig. 11), suggesting that Hs6st1 acts in synergy with Slit/Robo in this system and we confirmed this by analysis of Hs6st1+/−;Slit2+/− compound heterozygotes. Excitingly the Hs6st1+/−;Slit2−/− corollus phenotype revealed an unexpected interaction between Hs6st1 and Slit2. Rather than acting epistatically, as would be expected if Hs6st1 functioned to facilitate growth cone sensitivity to Slit2, our results can best be explained by Slit2 actually preventing corollus axons crossing the midline in Hs6st1+/− embryos. Our data fit a model where a primary function of Hs6st1 is to regulate the formation of Slit2-expressing glial structures and that the ectopic positioning of these glia in the path of corollus axons in Hs6st1+/− embryos blocks midline crossing. Regardless of the mechanistic details this case provides an example of a pathological axon guidance phenotype in which the defect can be treated by interfering with a second gene. In this case a defect caused by perturbation of sugar structure, via loss of a modifying enzyme, can be rescued by abrogating the function of the axon guidance molecule Slit2.

Robo/Slit and Hs2st mutants share a relatively mild disruption of midline glia and midline axon crossing defects with the formation of Probst-like axon bundles (Bagri et al., 2002; Andrews et al., 2006; López-Bendito et al., 2007) (Table 2; Fig. 11). On the other hand Hs2st and Robo/Slit mutants differ in (1) the proportion of corollus axons able to cross the midline, (2) in the penetrance of the axon guidance phenotype, and (3) Hs2st+/− corollus axons do not grow ventrally into the septum. Our examination of Hs2st+/−;Slit2+/− compound heterozygotes failed to identify, although does not rule out, a genetic interaction between Hs2st and Slit2. Overall our results suggest possible roles for Hs2st in some Robo/Slit functions but provides little indication of roles in additional pathways.

Our data points to primary functions for Hs6st1 in additional pathways to Slit/Robo. A good candidate is the FGF signaling pathway which, like Hs6st1, is critical for both axon navigation and the formation of midline glial structures (Fig. 11, compare K with M,N). HS is a component of the FGF/FGFR complex (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material) and the sulfation pattern of HS can dictate FGFR signaling and FGF-mediated axon guidance (Walz et al., 1997; DiGabriele et al., 1998; Guimond and Turnbull, 1999; Irie et al., 2002; Grobe et al., 2005). Telencephalic expression of FGFs or its receptor FGFR1 is critical for the generation of GW and IG glia and later knockout of FGFR1 restricted to the GFAP-expressing glia blocks their translocation from GW to IG (Smith et al., 2006; Tole et al., 2006; Moldrich et al., 2010). Corpus callosum axon midline crossing is blocked in all three models illustrating the importance of FGF signaling for the production of midline glial structures and subsequent corpus callosum development. We find that FGF signaling is reduced in the corticoSeptal region of Hs6st1−/− embryos at E14.5, glial differentiation in the GW is delayed so that there are very few mature glia in the GW at E16.5, and glia fail to populate the IG at E17.5. Together our results suggest a model in which Hs6st1 is required (1) for the normal developmental timetable of the glial structures required for corpus callosum formation and (2) for FGF-dependent events important in glial development.

The heparan sulfate code hypothesis

The heparan sulfate code hypothesis postulates that the interactions between HS and effector molecules involved in axon guidance, such as Slits or FGF molecules and their receptors, are dependent on the specific HS sugar structures generated by HS modifying enzymes (Turnbull et al., 2001; Bulow and Hobert, 2004; Holt and Dickson, 2005; Kreuger et al., 2006). In the present study we have taken advantage of mouse genetics to reveal the unique physiological functions for Hs2st and Hs6st1 in the development of two commissural axon tracts. Hs2st and Hs6st1 differ in both their biochemical action on HS sugar structure (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material) and in their gene expression patterns (Pratt et al., 2006; present study) so it is not possible to know, from the current study, the extent to which their unique functions rely on their biochemistry as opposed to their expression patterns. Future experiments involving more refined manipulation of Hs2st and Hs6st1 combined with glycomic technologies will help answer this important question.

References


