Heparan sulphate sulphation by Hs2st restricts astroglial precursor somal translocation in developing mouse forebrain by a non cell autonomous mechanism

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https://doi.org/10.1523/JNEUROSCI.1747-17.2018

Received: 22 June 2017

Revised: 5 December 2018

Accepted: 11 December 2018

Published: 7 January 2019

Author contributions: J.M.C., H.M.P., J.O.M., and T.P. designed research; J.M.C. and H.M.P. performed research; J.M.C. and H.M.P. analyzed data; J.M.C., H.M.P., and T.P. wrote the paper.

Conflict of Interest: The authors declare no competing financial interests.

We are grateful to the following people for supplying us with transgenic mouse lines: Hs2st\(^{fl}\), Jeffrey Esko (University College San Diego); Ext1\(^{fl}\), Yu Yamaguchi (Sanford Burnham Institute); Zic4\(^{Cre}\) and Emx1\(^{CreER}\), Nicoletta Kessaris (University College London). We would like to thank the anonymous reviewers whose comments have helped us improve the manuscript from its initial submission. This work was supported by grants from The Wellcome Trust (094832/Z/10/Z) and BBSRC (BB/M00693X/1) to TP and an EASTBIO BBSRC funded PhD studentship to HP.

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Cite as: J. Neurosci 2019; 10.1523/JNEUROSCI.1747-17.2018

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Abstract

Heparan sulphate (HS) is a cell surface and extracellular matrix carbohydrate extensively modified by differential sulphation. HS interacts physically with canonical fibroblast growth factor (FGF) proteins that signal through the extracellular signal regulated kinase 1 (ERK) pathway.
(ERK)/mitogen activated kinase (MAPK) pathway. At the embryonic mouse telencephalic midline FGF/ERK signalling drives astroglial precursor somal translocation from the ventricular zone of the cortico-septal boundary (CSB) to the induseum griseum (IG) producing a focus of Slit2-expressing astroglial guidepost cells essential for inter-hemispheric corpus callosum (CC) axon navigation. Here we investigate the cell and molecular function of a specific form of HS sulphation, 2-O HS sulphation catalysed by the enzyme Hs2st, in midline astroglial development and in regulating FGF protein levels and interaction with HS. Hs2st⁻/⁻ embryos of either sex exhibit a grossly enlarged IG due to precocious astroglial translocation and conditional Hs2st mutagenesis and ex vivo culture experiments show that Hs2st is not required cell autonomously by CC axons or by the IG astroglial cell lineage but rather acts non cell autonomously to suppress the transmission of translocation signals to astroglial precursors. Rescue of the Hs2st⁻/⁻ astroglial translocation phenotype by pharmacologically inhibiting FGF signalling shows the normal role of Hs2st is to suppress FGF-mediated astroglial translocation. We demonstrate a selective action of Hs2st on FGF protein by showing that Hs2st (but not Hs6st1) normally suppresses the levels of Fgf17 protein in the CSB region in vivo and use a biochemical assay to show Hs2st (but not Hs6st1) facilitates physical interaction between Fgf17 protein and HS.

Significance statement.

We report a novel non cell autonomous mechanism regulating cell signalling in developing brain. Using the developing mouse telencephalic midline as an exemplar we show that the specific sulphation modification of the cell surface and extracellular carbohydrate heparan sulphate (HS) performed by Hs2st suppresses the supply of translocation signals to astroglial precursors by a non cell autonomous mechanism. We further show that Hs2st modification selectively facilitates physical interaction between Fgf17 and HS and suppresses Fgf17 protein levels in vivo, strongly suggesting that Hs2st acts selectively on Fgf17 signalling. HS interacts with many signalling proteins potentially encoding numerous selective interactions important in development and disease so this class of mechanism may apply more broadly to other biological systems.

Introduction

The corpus callosum (CC) axon tract connects the cerebral hemispheres through the cortico-septal boundary (CSB) in mice and humans and CC malformation is associated with cognitive and neurological conditions in humans (Donahoo and Richards, 2009). Precisely
controlled radial glial cell (RGC) somal translocation from the ventricular zone (VZ) of the CSB to its pial surface generates midline zipper (MZ) and indium griseum (IG) astroglial populations required for cerebral hemisphere fusion and subsequent CC axon navigation (Clegg et al., 2014; Gobius et al., 2016; Inatani et al., 2003; Moldrich et al., 2010; Shu and Richards, 2001; Shu et al., 2003; Smith et al., 2006). The movement of RGC astroglial precursors from the glial wedge (GW) to the IG (GW→IG translocation) forms an astroglial guidepost population that secretes Slit2 to guide CC axons across the telencephalic midline.

Fibroblast growth factors (FGFs) are an evolutionarily ancient family comprising 23 genes in mice and humans of which 15 (Fgf1-10,16-18,20,22 in mice) encode ‘canonical’ FGFs that function as paracrine signalling molecules and bind promiscuously to cell surface FGF receptors (FGFRs encoded by Fgfr1-4 in mice) to elicit an extracellular signal regulated kinase (ERK)/mitogen activated kinase (MAPK) response via activating phosphorylation of ERK→phospo-ERK (pERK). Canonical FGFs are further subdivided into five subfamilies based on phylogeny and ‘Fgf8’ subfamily members Fgf8 and Fgf17 are transcribed in the developing CSB in close spatiotemporal proximity posing the question of how they are coordinated (Guillemot and Zimmer, 2011; Ornitz and Itoh, 2015). Under normal conditions GW→IG translocation is primarily attributed to Fgf8 and needs to be tightly regulated to ensure correct numbers of RGCs leave the GW and reach the IG. Deviation above (or below) normal FGF/ERK signalling levels induces too many (or too few) RGCs to translocate with consequent disruption to CC development (Clegg et al., 2014; Gobius et al., 2016; Smith et al., 2006; Wang et al., 2012). While Fgf17 plays a role in patterning the developing telencephalon its importance for CC development is less clear and no CC phenotype has been reported in Fgf17−/− embryos (Cholfin and Rubenstein, 2007, 2008). Because Fgf8 and Fgf17 are the principal Fgf’s transcribed in vicinity of the GW and both activate ERK, mechanisms must exist to keep the total amount of Fgf protein (Fgf8 protein + Fgf17 protein) at the correct level to generate the correct levels of ERK activation for astroglial precursor RGCs to translocate in appropriate numbers.

Heparan sulphate (HS), the carbohydrate component of cell surface and extracellular matrix (ECM) heparan sulphate proteoglycans, is a negatively charged sulphated polysaccharide that binds canonical FGFs in the ECM to regulate their movement and half-life and also functions as an obligate FGF co-receptor in FGF:FGFR:HS ternary signalling complexes on the cell surface (Balasubramanian and Zhang, 2016; Guillemot and Zimmer, 2011). HS biosynthesis is in two stages, Ext enzymes polymerise uronic acid – glucosamine disaccharides making linear [uronic acid – glucosamine]ₙ HS polymers which are then
modified by the enzymatic addition (by heparan sulphate sulphotransferases, HSTs) or removal (by heparan sulphate sulphatases, Sulfs) of sulphate groups at specific positions on the disaccharide residues. There are four classes of HST enzymes (Hs2st, Hs3st, Hs6st, and Ndst) each adding sulphate to a specific position, for example Hs2st only adds sulphate to the carbon atom in position 2 of uronic acid generating 2-O HS sulphation. While work in a variety of systems shows that HS itself can play roles both in the transmission of FGF signals through the ECM (non cell autonomous role) and the cellular response to FGF (cell autonomous role) the potential for specific forms of HS sulphation to selectively regulate FGFs by regulating the physical interaction between HS and FGF proteins is much less well understood (Allen et al., 2001; Allen and Rapraeger, 2003; Balasubramanian and Zhang, 2016; Belenkaya et al., 2004; Chan et al., 2015; Chan et al., 2017; Christian, 2012; Guillemot and Zimmer, 2011; Kinnunen et al., 2005; Loo et al., 2001; Loo and Salmivirta, 2002; Makarenkova et al., 2009; Qu et al., 2011; Qu et al., 2012; Ramsbottom et al., 2014; Toyoda et al., 2011; Yan and Lin, 2009; Yu et al., 2009; Zhang et al., 2012a).

The heparan sulphate code hypothesis states that different forms of HS sulphation can encode specific instructions (Kreuger et al., 2006; Turnbull et al., 2001). In this study we discover that 2-O HS sulphation catalysed by Hs2st functions non cell autonomously at the developing telencephalic midline to suppress FGF/ERK signalling that drives the somal translocation of astroglial precursors required for normal CC development and present evidence that Hs2st plays a selective role by modulating the physical interaction between Fgf17 protein and HS and selectively suppressing Fgf17 protein levels at the CSB.

Materials and Methods

Animals: All mice were bred in-house in line with Home Office UK legislation and licences approved by the University of Edinburgh Ethical Review Committees and Home Office. Embryos analysed in this study were of either sex. Animal husbandry was in accordance with UK Animals (Scientific Procedures) Act 1986 regulations. The Hs2st LacZ (Hs2st') null allele comprised a LacZ gene trap vector integrated into the Hs2st locus, the Hs6st1 LacZiresPLAP (Hs6st1') null allele comprised a LacZiresPLAP gene trap vector integrated in the Hs6st1 locus, both were genotyped by PCR as previously described (Bullock et al., 1998; Conway et al., 2011; Pratt et al., 2006). For some ex vivo experiments Hs2st'/' mice were crossed with mice carrying the TP6.3 Tau (τ) -GFP fusion transgene to generate Hs2st'/' and Hs2st'/' embryos with τGFP+' axons (Pratt et al., 2000). For conditional mutagenesis floxed
Ext1 (Ext1fl) or Hs2st (Hs2stfl) alleles were combined with either Zic4Cre (septal deletion) or Emx1CreER (cortical deletion) driver alleles (Inatani et al., 2003; Kessaris et al., 2006; Rubin et al., 2010; Stanford et al.). CreER activity was induced at E9.5 by administering tamoxifen (dissolved in corn oil using a sonicator) to pregnant dams by intraperitoneal (IP) injection (120mg/kg dose). Lineages of cells in which Cre was active were visualised using a Rosa26R-floxed-stop-EGFP reporter allele (Sousa et al., 2009).

**Ex vivo assays:** *Ex vivo* culture experiments were performed essentially as described previously (Niquille et al., 2009) Explants were cultured on nucleopore polycarbonate membranes (Whatman) floating on 1 ml ‘Neurobasal media’ (Neurobasal medium (Life Technologies) supplemented with L-glutamine, glucose and penicillin/streptomycin) at 37°C with 5% CO₂ in a humidified incubator. Brains were dissected from embryos in oxygenated Earle’s balanced salt solution (Life Technologies), embedded in low melting point agarose, sliced using a vibratome (Leica VTS-1000), and transferred to Modified Eagle medium (MEM, Life Technologies) with 5% foetal bovine serum for 1 hour. For CC axon navigation assays 400μm thick E17.5 coronal slices incorporating the CC axon tract were prepared and frontal cortex explants from τ-GFP⁺ slices were transplanted into the equivalent region in τ-GFP slices prior to culturing in Neurobasal media for 72 hours, fixation in 4% paraformaldehyde (PFA), and GFP immunofluorescence. For glial translocation experiments 10 mg/ml BrdU dissolved in PBS was injected IP into pregnant dams with E14.5 litters which were sacrificed 1 hour later and 350μm coronal slices incorporating the CSB prepared for culture. In Fgf17 bead experiments Affi-gel blue gel (Bio-rad) beads pre-soaked in 100μg/ml recombinant Fgf17 protein (R&D systems) or 5mg/ml BSA (Sigma) overnight at 4°C were implanted into the slice, one Fgf17 and one BSA bead on either side of the midline just below the GW, and the MEM replaced with Neurobasal media. For the FGFi culture, MEM media was replaced with Neurobasal media containing either 25μM SU5402, 0.1% DMSO (FGF inhibitor (FGFi) treated) or 0.1% DMSO (control). Slices were cultured for 2 or 48 hours, fixed in 4% PFA, and 10μm frozen sections prepared for immuno-detection or *in situ* hybridisation. Glial migration out of the VZ towards the pial surface was quantified from BrdU/Sox9 immunofluorescence micrographs by demarcating the basal edge of the VZ (easily identified by Sox9 staining) with a line and counting the number of Sox9⁺;BrdU⁺ cells which had crossed this line. This allowed us to count glial (Sox9⁺) cells that had incorporated BrdU (BrdU⁺) when they were in the VZ before the start of the culture and subsequently exited the VZ and migrated towards the midline over the 2 day culture period when the
cultures were exposed to experimental substances (SU5402, DMSO, Fgf17 protein, or BSA). 4 or 6 sections were quantified per slice moving rostrally from the most caudal section in which the GW could be identified on both sides of the section.

**Immunodetection:** Embryonic mouse brains were removed and fixed in 4% PFA in PBS overnight at 4°C, cryoprotected in 30% sucrose in PBS, embedded in OCT, and 10μm coronal frozen sections cut using a cryostat (Leica). Immunohistochemistry was performed as described previously (Clegg et al., 2014). Primary antibodies: goat anti-GFP (diluted 1/250, Abcam); rabbit anti-Sox9 (1/500, Cell Signalling Technologies); rat anti-L1 (1/200, Millipore); rabbit anti-GFAP (1/200 Dako); rabbit anti-Hs2st (1/50, Abcam ab103120); rabbit anti-Fgf17 (1/1000, Abcam ab187982); and rabbit anti-pErk1/2 (1/200, Cell signalling).

Secondary antibodies; donkey anti-goat Alexa Fluor 488; donkey anti-rabbit Alexa Fluor 568; and goat anti-rat 568 (all used at a dilution of 1/200 and from Invitrogen). Fluorescently labelled sections were counterstained with DAPI (Invitrogen). For Hs2st and pErk1/2 antibody staining goat anti-rabbit biotin secondary antibody (1/200, Vector Laboratories) was used and staining was visualised using a standard avidin-biotin daiminobenzidine (DAB) staining procedure. The Fgf17 immunofluorescence was performed using exactly the same protocol as previously described for Fgf8 except that the Fgf8 antibody was replaced with the Fgf17 antibody (Clegg et al., 2014; Toyoda et al., 2011). Briefly, slides were first washed in acetone for permeabilisation, Rabbit Fgf17 antibody applied, and the TSA plus Fluorescence System kit (Perkin Elmer) used for fluorescence detection.

**In situ Hybridisation:** In situ hybridisation was carried out on 10μm frozen sections as previously described (Wallace and Raff, 1999) using digoxigenin-labelled riboprobes for Slit2 and Fgf17 (Erskine et al., 2000; Xu et al., 1999).

**Imaging:** Fluorescent labelled sections were imaged using either a Leica AF6000 epifluorescence microscope coupled to a Leica DFC360 digital camera or a Nikon Ti: E Inverted confocal microscope. DAB stained and in situ hybridised sections were imaged using a Leica DLMB microscope coupled to a Leica DFC480 colour digital camera.

**Fgf17 protein quantification:** Fgf17 fluorescence was quantified from E14.5 Hs2st+/+;Hs6st1+/+, Hs2st-/- and Hs6st1-/- coronal sections that had been processed for Fgf17 immunofluorescence in parallel and imaged under identical conditions in parallel using the same method as previously described for Fgf8 protein quantification (Chan et al., 2017). For each section IMAGE J was used to measure mean fluorescence intensity in a 100 x 150 μm
box drawn at the CSB encompassing the Fgf17 expression domain. For each embryo quantification was performed for three sections along the rostro-caudal axis and averaged.

**IG Sox9⁺ cell quantification:** Quantification of Sox9 immunofluorescent positive cells (Sox9⁺ cells) in the IG region of E18.5 Hs2st⁰/⁺;Zic4Cre, Hs2st⁺/+;Zic4Cre Hs2st⁰/⁺;Emx1CreER, and Hs2st⁺/+;Emx1CreER embryos were performed as previously described (Clegg et al., 2014). A counting box measuring 200 μm x 200 μm was placed on images of coronal sections at the midline with the top edge at the dorsal extent of Sox9⁺ cells at the IG and the numbers of Sox9⁺ cells in the box counted. For each embryo quantification was performed for three sections along the rostro-caudal axis and averaged.

**Western Blotting:** Western blotting was performed as previously described (Clegg et al., 2014), primary antibodies: rabbit anti-Hs2st (1/500, Abcam ab103120) and mouse anti-β-actin (1/5000, Abcam). Secondary antibodies: goat anti-mouse Alexa Fluor 680 (Invitrogen) and goat anti-rabbit 800 (Li-Cor).

**Ligand and Carbohydrate Engagement (LACE) Assay:** LACE assay was performed as previously described (Allen et al., 2001; Allen and Rapraeger, 2003; Chan et al., 2015).

Briefly, frozen sections were incubated in 0.05% NABH₄/PBS for 15 min. After several washes in PBS, sections were incubated in 0.1M glycine at 4°C overnight. Some sections were incubated with Heparitinase I (Seikagaku) before proceeding. All Fgf and Fgfr-Fc proteins were purchased from R&D Systems. Sections were then treated with 1% BSA/TBS solution for 10 min before incubation with 3 μM recombinant mouse Fgf17 and 9 μM recombinant human Fgfr1a(IIIc)-Fc or 30nM recombinant mouse Fgf8b and 100nM recombinant human Fgfr3 (IIIc)-Fc at 4°C overnight. Fgf17 or Fgf8 were omitted from some assays. Fluorescent LACE signal was generated by incubation with 1/200 anti-human IgG (Fc-specific) Cy3 (Sigma) in 1% BSA/TBS. Hs2st⁺/+;Hs6st1⁺/+; Hs2st⁻/⁻ and Hs6st1⁻/⁻ material that had been processed for each LACE assay condition in parallel were imaged under identical conditions in parallel. For each section IMAGE J was used to measure mean fluorescence intensity in a 100 x 150 μm box drawn encompassing the CSB. Background signal was quantified from control LACE experiments from which the FGF ligand was omitted and these values used for background subtraction. For each embryo quantification was performed for three sections along the rostro-caudal axis and averaged.

**Data analysis and statistics:** Results are expressed as mean ±SEM. The statistical test and sample size (n) for each experiment are specified in the figure legends. Statistical comparison...
between 2 groups was performed with a t-test. Statistical comparison between > 2 groups was performed with ANOVA followed by post-hoc t-test. p < 0.05 was considered significant.

Results

Hs2st protein is widely expressed in the developing cerebral cortex and at the telencephalic midline.

In order to establish potential sites of action of Hs2st in CC development we first examined the distribution of cells expressing Hs2st protein and contributing to developing CC structures using Hs2st immunohistochemistry at E14.5 (Fig 1 A-D) and E18.5 (Fig 1 E-M) spanning the period of CC axon tract development. Macroscopically, Hs2st protein distribution closely resembles the Hs2st-LacZ reporter staining previously reported with widespread Hs2st expression in the developing cerebral cortex and at the CSB at both E14.5 and E18.5 (Fig 1A,E, boxed areas indicate regions shown at higher magnifications in B-D and F-M) (Conway et al., 2011). Subcellularly the Hs2st signal is punctate consistent with the expected localisation of Hs2st in the Golgi apparatus (arrows point to Hs2st+ puncta in higher magnification insets in Fig 1B,F,P). At E14.5 there was a high density of Hs2st+ puncta at the CSB in the GW region where IG astroglial RGC precursors reside (Fig 1B with boxed area shown as higher magnification inset with arrows indicating Hs2st+ puncta) with the density falling towards the pial surface although Hs2st+ puncta were visible. There were many Hs2st+ puncta in the VZ of the cerebral cortex (Fig 1C) and also in the cortical plate (Fig 1D) indicating that many cortical progenitors and post-mitotic neurones express Hs2st. At E18.5 Hs2st is expressed by many cells in the IG (Fig 1F) and at the apical surface of the ventricular zone (VZ) at the GW (Fig 1G), septum (Fig 1H), and ventral telencephalon (Fig 1I) with the number of Hs2st expressing VZ cells diminishing as distance from the ventricle increases. In the cerebral cortex Hs2st is expressed by many cells close to the apical surface of the VZ (Fig 1J). Large numbers of post-mitotic cortical neurons outside the VZ express Hs2st and moving towards the pial surface the density of Hs2st+ puncta varies with laminar position (compare Fig 1 K,L,M showing relatively high Hs2st+ puncta density in cortical layers adjacent to the pial membrane (M) and in the intermediate zone (K) and lower density in the intervening region (L)). We validated the Hs2st antibody by demonstrating absence of the punctate Hs2st+ immunostaining in Hs2st−/− embryonic material (compare Fig 1N,P to O,Q – note that the more diffuse staining persists in Hs2st−/− tissue and we discounted this as non-specific background) and western blot showing that the predicted 42kDa Hs2st protein band was present in Hs2st+/+ and absent from Hs2st−/− telencephalic protein extracts (Fig 1R).
conclude, Hs2st protein is present in developing cerebral cortex, the source of CC axons, as well as in progenitor and post-mitotic cells of the CSB region constituting the environment through which midline crossing CC axons navigate. The Hs2st expression analysis suggests multiple potential sites of action for 2-O HS sulphation in CC development.

**The Slit2 expressing IG is expanded in Hs2st<sup>−/−</sup> embryos.**

We previously reported that increased numbers of astroglia at the pial surface of the Hs2st<sup>−/−</sup> CSB stemmed from precocious glial translocation and found no evidence that changes in cell proliferation or death contributed to this phenotype (Clegg et al., 2014; Conway et al., 2011). In order to determine whether there is an expansion of the IG in Hs2st<sup>−/−</sup> embryos we compared the expression of Slit2 mRNA, a marker of GW and IG glia but not MZ glia, between Hs2st<sup>+/+</sup> and Hs2st<sup>−/−</sup> embryos at E16.5 (Shu and Richards, 2001; Shu et al., 2003). In Hs2st<sup>+/+</sup> embryos Slit2<sup>+</sup> cells form a compact focus at the IG that increases in size moving caudally (Fig 2 A,C,E – Slit2 expression domain at IG indicated by brackets). In Hs2st<sup>−/−</sup> embryos the Slit2 expression domain is greatly expanded at the pial surface along the rostro-caudal axis (Fig 2 B,D,F – expanded Slit2 expression domain indicated by brackets). We conclude that an expansion of the Slit2<sup>+</sup> IG astroglial population makes a major contribution to the Hs2st<sup>−/−</sup> phenotype.

**Cell autonomy of HS and 2-O HS sulphation in astroglial precursor somal translocation and corpus callosum development.**

We next exploited conditional mutagenesis of Hs2st or Ext1 to experimentally uncouple specific functions of 2-O sulphation from more general functions of HS in astroglial precursor translocation and corpus callosum development. Widespread expression of HS and 2-O HS sulphation leaves open the possibility that each regulates GW→IG astroglial precursor somal translocation cell autonomously by modulating the response to signals, non cell autonomously by regulating the supply of signals, or both. To resolve this we identified two Cre alleles, Zic4<sub>Cre</sub> and Emx1<sub>CreER</sub>, that drive LoxP mediated mutagenesis in the astroglial lineage or in their cellular environment respectively and used them to conditionally ablate either HS (Ext1<sup>loxP</sup> mutagenesis) or 2-O HS sulphation (Hs2st<sup>loxP</sup> mutagenesis) to test for cell autonomous or non cell autonomous functions. We refer to these as ‘Zic4 lineage’ and ‘Emx1 lineage’ and next present their characterisation using a floxed-stop GFP reporter that turns on GFP expression in Cre expressing cells and their descendants before describing experiments where they are employed to conditionally generate loss of function mutations in...
Ext1Fl or Hs2stFl alleles (Inatani et al., 2003; Kessaris et al., 2006; Rubin et al., 2010; Sousa et al., 2009; Stanford et al.).

Characterisation of Zic4 and Emx1 lineages.

The septum is of Zic4 lineage, as shown by strong expression of the GFP reporter (Fig 3A). The GFP signal in the intermediate zone of the cerebral cortex (asterisks, Fig. 3A) is due to GFP+ thalamocortical axons that project from Zic4 lineage cells in the thalamus and cells of sub-cortical origin as previously reported (Rubin et al., 2010). At the midline GFP+ cells of the Zic4 lineage are predominantly located ventral to the CSB (dashed lines in Fig 3B) but there is also GFP expression in the IG (boxed area ‘D’ in Fig 3B). Sox9 is a transcription factor that marks the nuclei of RGCs in the VZ and differentiated astroglia in the IG and MZG and we previously showed that the positioning of Sox9+ cells is of critical importance for the development of the CC (Clegg et al., 2014). Combining GFP with Sox9 immunostaining reveals the contribution of the Zic4 lineage to the CSB astroglial populations. There is a sharp boundary (dashed line in Fig 3C) in the VZ of the CSB between Sox9+;GFP+ cells (arrowheads in Fig 3C) on the septal side and Sox9+;GFP- cells (arrows in Fig 3C) on the cortical side. Virtually all the Sox9+ cells in the IG (Fig 3D) and MZG (fig 3E) are also GFP+ (arrowheads in Fig 3D,E) indicating that these cells are Zic4 lineage. These data show that the Zic4 lineage contributes Sox9+ cells to the septal VZ and strikingly is the sole source of Sox9+ astroglia in the IG (Fig 3F).

To mark the Emx1 lineage, tamoxifen was administered to Emx1CreER embryos harbouring the floxed-stop GFP reporter at E9.5 so that early Emx1 expressing cerebral cortex progenitors and their descendants were rendered GFP+. Examination of the expression of the GFP reporter shows that, as expected, the developing cerebral cortex and CC axons are of Emx1 lineage (Fig. 3G) and that at the midline GFP expression is predominantly located dorsal to the CSB (dashed lines in Fig 3H). Higher magnification shows that there is a sharp boundary between GFP+ and GFP- cells at the VZ of the CSB (dashed line in Fig 3I). Combining Sox9 and GFP immunostaining reveals the contribution of the Emx1 lineage to Sox9+ cells. Sox9+;GFP+ cells (arrowheads in Fig 3I) populate the VZ on the cortical side of the boundary with Sox9+;GFP- cells on the septal side (arrows in Fig 3I) showing that the Emx1 lineage contributes Sox9+ cells exclusively to the cortical side of the VZ. All Sox9+ cells in the IG (Fig 3J) and MZ (Fig 3K) are GFP- (arrows in Fig 3J,K) indicating that the Emx1 lineage does not contribute Sox9+ cells to the IG. These data show that the Emx1
lineage contributes Sox9+ cells to the cortical VZ but no cells of this lineage contribute Sox9+ astroglia to the IG (schema in Fig 3L).

**Ext1** is required by both **Emx1** and **Zic4** lineage cells for corpus callosum development.

To determine the cellular requirement for HS we deleted *Ext1*, essential for HS synthesis, in the **Zic4** or **Emx1** lineages. In control embryos L1 immunostaining labels axons in the U-shaped CC while GFAP staining labels midline astroglial structures (Fig. 4A with higher magnification of IG and GW in Fig 4D,G). Removing HS from either the **Zic4** lineage (Fig 4B with higher magnification of IG and GW in Fig 4E,H) or the **Emx1** lineage (Fig 4C with higher magnification of IG and GW in Fig 4F,I) generates a severe CC agenesis phenotype (*Zic4Cre;Ext1Fl/Fl* n=4/4; *Emx1CreER;Ext1Fl/Fl* n=3/3). In **Emx1** conditional mutants (*Emx1CreER;Ext1Fl/Fl*) CC axons fail to cross the midline and form Probst bundles (P) some distance short of the midline (Fig. 4B). GFAP+ astroglial cells are present in the IG (Fig 4E) and at the GW (fig 4H) in a pattern grossly similar to that of controls (compare Fig. 4D,G to E,H). In **Zic4** conditional mutants (*Zic4 Cre;Ext1Fl/Fl*) CC axons approach the midline but fail to cross (Fig. 4C with higher magnification of IG and GW in Fig 4F,I). Astroglial populations in **Zic4** conditional mutants are obviously disrupted with less intense GFAP staining at the midline (compare IG region in Fig 4 D to F) and more GFAP at the GW than in controls (arrows in Fig 4I, compare Fig. 4G to I) suggesting that in these embryos astroglial precursors translocate less efficiently to the IG and instead remain in the GW. We noted that the cerebral cortex of *Zic4Cre;Ext1Fl/Fl* brains was thinned and the ventricles were enlarged (compare Fig4 A to C), this hydrocephalus-like phenotype is intriguing because the cerebral cortex is not of the **Zic4** lineage indicating a non-cell autonomous mechanism by which HS regulates cerebral cortex development. The FGFR1/FGF2 ligand and carbohydrate engagement (LACE) assay detects endogenous HS on tissue sections by forming ternary complexes with exogenously added FGF2 and FGFR1 (red LACE signal in Fig 4 J-O) (Allen et al., 2001; Chan et al., 2015). HS is ubiquitously expressed in both cortical and septal compartments of control telencephalon (Fig 4J, higher magnification of CSB in M) and, as intended, HS synthesis is blocked in the cortex and cortical axons of *Emx1CreER;Ext1Fl/Fl* embryos (Fig 4K, CSB shown at higher magnification in N with arrows indicating HS deficient cortical region) and in the septum of *Zic4Cre;Ext1Fl/Fl* embryos (Fig 4L, higher magnification of CSB in O with arrows indicating HS deficient septum). Predigesting tissue sections with heparitinase eliminated the LACE signal (not shown) confirming specificity of this assay for detecting HS. The salient conclusions from the *Ext1* conditional mutagenesis
for the current study are that HS is indispensable from both the Zic4 and the Emx1 lineages for CC development and that removing HS from the Zic4 lineage inhibits Zic4 lineage astroglia reaching the IG region.

**Hs2st is required by Emx1 lineage but not Zic4 lineage cells for corpus callosum development.**

Having established that both Zic4 and Emx1 lineages need to synthesise HS for normal CC development we next asked whether 2-O HS sulphation of the HS is required in either lineage. Hs2st is the sole enzyme capable of imparting 2-O HS sulphation onto HS so to determine the cellular requirement for 2-O HS sulphation we deleted Hs2st in the Zic4 or Emx1 lineages. Control Hs2st^{+/+} genotypes (Hs2st^{+/+};Emx1^{CreER} and Hs2st^{+/+};Zic4^{Cre}) displayed neither CC agenesis nor midline astroglial disorganisation, the control embryo shown in Fig 5 (A,D,G,J – D,G reproduced from Fig 3 H,J) is Hs2st^{+/+};Emx1^{CreER} genotype. The CC and the midline astroglial structures form normally in Hs2st^{fl/fl};Zic4^{Cre} conditional mutants and the organisation of L1^{+} axons and GFAP^{+} astroglia are indistinguishable from control embryos (6/6 embryos) (Fig. 5B, compare to control in 5A). The organisation of GFP^{+} Zic4 lineage cells is the same in Hs2st^{fl/fl};Zic4^{Cre} embryos as in Hs2st^{+/+};Zic4^{Cre} embryos (compare Fig 5E,H to Fig 3B,D) and IG Sox9^{+} cell counts confirm that the numbers of Sox9^{+} cells in the IG are not significantly different to control Hs2st^{+/+} embryos (Fig 5M, compare blue and orange bars) indicating no cell autonomous requirement for Hs2st in the Zic4 lineage Sox9^{+} IG astroglia. To exclude the possibility of a compensatory mechanism by which the Hs2st^{fl/fl};Zic4^{Cre} IG is populated by Hs2st^{+/+} cells from a different lineage we performed Hs2st immunohistochemistry and confirmed that Hs2st expression is indeed absent from all cells in the IG (Fig 5K, note this is an adjacent section from the same embryo to the one shown in 5H). In Hs2st^{fl/fl};Emx1^{CreER} embryos the CC fails to form in approximately 50% of cases, embryos either had a severe phenotype (Fig 5C, 5/9 embryos) or appeared completely unaffected (Fig 5C', 4/9 embryos). CC axons form Probst bundles (P) on either side of the telencephalic midline while the GFAP^{+} IG is expanded (asterisks, Fig. 5C). The anatomy and incomplete penetrance of the CC phenotype in Hs2st^{fl/fl};Emx1^{CreER} embryos closely resemble constitutive null Hs2st^{+/} embryos indicating that Hs2st function within the Emx1 lineage is sufficient for normal CC development (Clegg et al., 2014; Conway et al., 2011). As in control Hs2st^{+/+};Emx1^{CreER} embryos (Fig 5 D,G) the GFP and Sox9 signals did not overlap in the IG region of control or Hs2st^{fl/fl};Emx1^{CreER} embryos (Fig 5F, boxed area shown at higher magnification in 5I) and the Sox9^{+} cells in the IG of control
embryos and the expanded IG of Hs2st<sup>fl/fl</sup>;Emx1<sup>CreER</sup> embryos were GFP− (arrows in Fig 5I indicate Sox9<sup>+</sup>;GFP− cells). Counts of Sox9<sup>+</sup> cells confirmed a significant increase in the IG of affected Hs2st<sup>fl/fl</sup>;Emx1<sup>CreER</sup> embryos compared to controls (Fig 5M, compare blue and purple bars). Immunostaining for Hs2st on adjacent sections confirmed that IG cells in Hs2st<sup>fl/fl</sup>;Emx1<sup>CreER</sup> embryos retain Hs2st protein expression (Fig 5L). Because Sox9<sup>+</sup> IG astroglia do not belong to the Emx1 lineage their ectopic position in Hs2st<sup>fl/fl</sup>;Emx1<sup>CreER</sup> embryos, despite retaining Hs2st function, allows us to conclude a cell non autonomous requirement for Hs2st in the translocation of astroglial precursors to the IG.

The salient conclusions from these conditional mutagenesis experiments are that while the Zic4 lineage astroglia do require Ext1 to form midline astroglial structures they do not require Hs2st, strongly suggesting that while these Zic4 lineage cells require HS on their cell surface to respond to translocation signals there is no need for the HS to be 2-O sulphated. In contrast Hs2st is absolutely required in the surrounding Emx1 lineage cells indicating a non cell autonomous mechanism by which 2-O HS sulphation controls the transmission of translocation signals to the Zic4 lineage astroglial precursors.

**Hs2st is not required cell autonomously by CC axons to navigate the midline.**

The conditional mutagenesis experiments showed that Hs2st has a non cell autonomous role in GW→IG somal translocation, but, because Hs2st is expressed throughout the cerebral cortex, did not resolve whether there is an additional cell autonomous requirement in CC axon navigation. To answer this we performed ex vivo transplant experiments in which cerebral cortical tissue from transgenic mice ubiquitously expressing τGFP, which efficiently labels axons of τGFP<sup>+</sup> cells, was transplanted into τGFP<sup>−</sup> telencephalic slices containing the CC axon pathway and CSB structures (Niquille et al., 2009; Pratt et al., 2000). When wild-type (WT) E17.5 τGFP<sup>+</sup> cortical explants are transplanted into age matched τGFP<sup>−</sup> WT cortical slices τGFP<sup>+</sup> axons extend across the telencephalic midline forming the characteristic U-shape of the CC and reach the cortex of the opposite hemisphere (n=3/3 cultures, arrows in Fig 6A,D point to crossing axons). When Hs2st<sup>−/−</sup> τGFP<sup>+</sup> cortical explants are transplanted into τGFP<sup>−</sup> WT slices, axons are able to cross the midline to reach the opposite hemisphere in a manner indistinguishable from that seen in the WT→WT transplants (n=4/4 cultures, arrows in Fig 6B,E point to crossing axons). In contrast, when τGFP<sup>+</sup> WT cortical explants are transplanted into τGFP<sup>−</sup> Hs2st<sup>−/−</sup> slices axons are unable to reach the opposite cortical hemisphere and instead remain within the cingulate cortex or invade the septum (n=6/6 cultures), resembling the in vivo CC phenotype observed
in Hs2st\(^{-/-}\) embryos (Clegg et al., 2014; Conway et al., 2011). Note that in all cultures a few axons grew into the septum (arrowheads in Fig 6 D,E,F). Schematics summarising these experiments are shown in Fig 6 G,H,I. These data show 2-O HS sulphation is not required cell autonomously by CC projection neurons for axon guidance across the midline strongly suggesting that disorganisation of midline guidepost astroglial cells is the primary cause of the Hs2st\(^{+/}\) CC agenesis phenotype.

Abnormally high FGF/ERK signalling causes the Hs2st\(^{+/}\) precocious astroglial translocation phenotype.

We previously reported a correlation between hyperactive ERK signalling at the CSB and precocious somal translocation of astroglia to the midline in Hs2st\(^{+/}\) embryos but we did not formally establish that this stemmed from hyperactive FGF/ERK signalling (Chan et al., 2017; Clegg et al., 2014). To address this we employed an ex vivo assay in which coronal WT or Hs2st\(^{+/}\) telencephalic slices incorporating the CSB were cultured on floating membranes for long enough to allow somal translocation to the midline and attempted to rescue the Hs2st\(^{+/}\) phenotype by pharmacological abrogation of FGF/ERK signalling. WT or Hs2st\(^{+/}\) E14.5 slices were cultured in the presence of the Fgfr1 inhibitor SU5402 dissolved in DMSO (FGFi treatment) to inhibit FGF/ERK signalling or in DMSO alone (untreated control) for 48hr (Fig 7A). To aid subsequent identification of translocating cells, a subpopulation RGCs undergoing S-phase in the VZ at E14.5 were labelled just prior to culturing with a single pulse of BrdU. Immunohistochemistry for pErk (brown stain in Fig 7 B,D,F,H) confirms inhibition of FGF/ERK signalling in both FGFi treated WT and Hs2st\(^{+/}\) cultures (Fig 7D,H) compared to untreated cultures (Fig 7B,F) showing that FGF signalling through Fgfr1 accounts for ERK phosphorylation in both genotypes so, importantly, demonstrating that ERK hyperactivation in Hs2st\(^{+/}\) embryos does not stem from an FGF-independent mechanism for ERK activation (Chan et al., 2017; Clegg et al., 2014). After 48 hours some Sox9\(^{+}\) cells (red) had left the VZ and translocated to the midline in untreated WT cultures (arrow in Fig 7C) with many more populating the midline in untreated Hs2st\(^{+/}\) cultures (arrow in Fig 7G) validating that our ex vivo assay replicates the in vivo Hs2st\(^{+/}\) phenotype. Consistent with our hypothesis, FGFi treatment of both WT and Hs2st\(^{+/}\) cultures resulted in a large decrease in Sox9\(^{+}\) cells reaching the midline (compare Fig 7E,I to C,G). We quantified glial translocation by counting the numbers of Sox9\(^{+}\) cells born in the VZ at E14.5 (Sox9\(^{+}\);BrdU\(^{+}\) cells, yellow – inset in Fig 7 C,E,G,I shows higher magnification) that had exited the VZ towards the midline (VZ demarcated by dotted line in Fig 7 C,E,G,I) after 2 days in culture. Counts of
BrdU⁺;Sox9⁺ cells showed that glial translocation was significantly greater in *Hs2st<sup>−/−</sup>* compared to WT cultures along the rostro-caudal axis (dark purple and green lines in Fig 7J) and in both cases almost completely suppressed by FGFi treatment (pale purple and green lines in Fig 7J).

We conclude that the precocious glial translocation phenotype in *Hs2st<sup>−/−</sup>* embryos is caused by hyperactive FGF/ERK signalling from E14.5 onwards. Taken together with our *Hs2st* conditional mutagenesis experiments demonstrating a non cell autonomous role for *Hs2st* in astroglial precursor translocation we hypothesise that *Hs2st* normally suppresses the supply of FGF proteins to translocation competent astroglial precursors in the GW.

**Hs2st suppresses Fgf17 protein levels.**

We next sought to identify an FGF protein that is targeted by *Hs2st*. Despite its well known role in CC development Fgf8 protein levels are not significantly increased at the CSB of *Hs2st<sup>−/−</sup>* embryos forcing us to consider other FGFs (Chan et al., 2017; Clegg et al., 2014). A promising candidate is *Fgf17*, a member of the *Fgf8* subfamily transcribed at the CSB in a similar pattern to *Fgf8* (Cholfin and Rubenstein, 2008; Zhang et al., 2012b). *Fgf17* is a canonical FGF that binds to HS, so is potentially regulated via its interaction with HS, and is known to play a role in patterning the telencephalon although its role in CC development has not been fully characterised (Cholfin and Rubenstein, 2007; Hoch et al., 2015; Li and Kusche-Gullberg, 2016). We hypothesised that *Hs2st* normally suppresses *Fgf17* protein and predicted that *Fgf17* protein levels would be increased at the *Hs2st<sup>−/−</sup>* CSB. We compared the expression of *Fgf17* protein in the developing CSB of WT and *Hs2st<sup>−/−</sup>* embryos at three developmental stages (E12.5, E14.5, and E16.5), spanning the interval of midline glial translocation (Fig 8A1-2, H1-2). At E12.5 telencephalic *Fgf17* protein is restricted to the CSB region with no obvious difference between WT and *Hs2st<sup>−/−</sup>* (compare Fig 8A1, B1 to A2, B2). By E14.5 there is an expanded *Fgf17* protein domain at the CSB of *Hs2st<sup>−/−</sup>* embryos (compare Fig 8D1, E1 to D2, E2, * in E2 marks the expanded *Fgf17* protein domain). Quantification of *Fgf17* immunofluorescence shows a significant ~2 fold increase in *Fgf17* protein levels in this region of *Hs2st<sup>−/−</sup>* CSB (Fig 8W – compare blue and green bars). At E16.5 *Fgf17* protein is much closer to detection threshold than at the earlier stages in both genotypes (Fig 8G1,2, H1,2) although the increased protein spread in the mutant persists (*) in Fig 8H2 indicating that the *Hs2st<sup>−/−</sup>* CSB is exposed to a prolonged overdose of *Fgf17* protein spanning E14.5-E16.5. We next examined *Fgf17* mRNA at the CSB so see whether the
increase in Fgf17 protein in \( Hs2st^{-/-} \) CSB was underpinned by altered Fgf17 gene expression. There was no evidence for this at E12.5 or E14.5 where Fgf17 mRNA expression pattern remains similar between \( Hs2st^{+/+} \) and \( Hs2st^{-/-} \) embryos (compare Fig 8 C1 to C2 and F1 to F2), however, the expression domain of Fgf17 mRNA is increased in E16.5 \( Hs2st^{-/-} \) CSB (compare Fig 8I1 to I2, * in I2 marks expanded Fgf17 mRNA domain). This subsequent increase in Fgf17 mRNA in the E16.5 \( Hs2st^{-/-} \) CSB indicates that the \( Hs2st^{-/-} \) phenotype has a transcriptional component or that there are more cells expressing Fgf17 mRNA in the expanded \( Hs2st^{-/-} \) IG although this cannot be the primary event as it is not apparent at E14.5, the stage at which we previously identified precocious astroglial precursor translocation was well underway in \( Hs2st^{-/-} \) embryos (Clegg et al., 2014).

Mosaic analysis (Fig 5) indicated that \( Hs2st \) function in the \( Emx1 \) lineage negatively regulates a signal promoting GW→IG translocation of Zic4 lineage glial cells by a non cell autonomous mechanism and Fgf17 expression analysis (Fig 8) makes Fgf17 a strong candidate for the signal. Based on this we hypothesised that Fgf17 is expressed in cells surrounding the Zic4 lineage cells and performed detection of Fgf17 mRNA or protein in E14.5 WT embryos in which the Zic4 lineage is labelled GFP+. Fgf17 mRNA is expressed at the GW and the IG (Fig 8J) and higher power magnification shows that in the VZ GFP+ cells express little if any Fgf17 mRNA and conversely cells expressing the highest levels of Fgf17 mRNA are GFP+ (Fig 8 K1,3, arrows indicate GFP+ cell location). This complementarity between Fgf17 mRNA expressing and Zic4 lineage cells is preserved at the IG (Fig 8L1,3, arrows indicate GFP+ cell location). Fgf17 protein predominates at the IG (Fig 8M) and higher power magnification shows that while Fgf17 protein is barely detectable at the GW (Fig 8N1,3) there are a number of much higher Fgf17 expressing cells at the IG and these cells are GFP confirming that they do not belong to the Zic4 lineage (Fig 8O1,3, arrows indicate GFP+ cell location). Interestingly, although cells in the GW and IG express comparable levels of Fgf17 mRNA (compare Fig 8K1 to L1) the expression of Fgf17 protein is much higher in the IG (compare Fig 8N1 to O1) suggesting a post-transcriptional repression selectively at the GW. Our identification of Hs2st as a repressor of Fgf17 protein levels at this stage makes Hs2st a strong candidate, indeed, closer examination of \( Hs2st \) expression using the \( Hs2st\)-LacZ reporter shows that Hs2st is expressed in a \( \text{GW}^{\text{High}}\text{-IG}^{\text{Low}} \) pattern (Fig 8P, also apparent in the Hs2st immunohistochemistry (Fig 1B)), complementary to the \( \text{GW}^{\text{Low}}\text{-IG}^{\text{High}} \) Fgf17 protein distribution. Together these data bolster the idea that Hs2st acts to suppress Fgf17 protein supply to Zic4 lineage cells by a post-transcriptional mechanism.
We conclude that Hs2st primarily suppresses the level and spread of Fgf17 protein emanating from the Emx1 lineage in the CSB.

**Hs6st1 does not affect Fgf17 protein levels.**

We next addressed whether the ability of 2-O HS sulphation to suppress Fgf17 protein levels in vivo represented a specific function of Hs2st or was redundant with other HSTs. We chose to examine Hs6st1, an HST that catalyses 6-O HS sulphation, because we have previously shown that Hs6st1 (but not Hs2st) suppresses levels of the closely related Fgf8 protein at the CSB in vivo (Chan et al., 2017; Clegg et al., 2014). However, we were unable to detect increased expression of Fgf17 protein (compare Fig 8 Q1, R1 to Q2, R2 and T1, U1 to T2, U2) or Fgf17 mRNA (compare Fig 8S1 to S2 and V1 to V2) in Hs6st1−/− compared to wild-type CSB at either E14.5 or E16.5. Quantification of Fgf17 immunofluorescence shows unchanged Fgf17 protein levels in this region of Hs6st1−/− CSB (Fig 8W – compare blue and purple bars). These data demonstrate that the negative relationship between Hs2st and Fgf17 is selective in vivo because it does not apply to Hs6st1.

**Exogenously applied Fgf17 phenocopies the Hs2st−/− astrogial translocation phenotype.**

Our data suggest that the Hs2st−/− phenotype stems from abnormally high levels of Fgf17 protein at the CSB causing FGF/ERK hyperactivation and precocious somal translocation to the IG. This requires that Hs2st−/− CSB cells are competent to respond to Fgf17 protein and that application of ectopic Fgf17 triggers precocious glial translocation, neither of which have been previously established. We redeployed the CSB ex vivo culture assay (see Fig 7) with the modification that beads soaked in either recombinant Fgf17 protein (Fgf17 treatment) or in BSA (control) were implanted into coronal slices of CSB region on either side of the midline (Fig 9A). WT or Hs2st−/− slices implanted with Fgf17 and BSA beads were cultured for 2 hours before processing for Fgf17 (green signal) and pErk (red signal) double immunofluorescence (Fig 9B). In both WT and Hs2st−/− cultures Fgf17 protein was detectable adjacent to the edge of the bead (green signal) and this activated ERK phosphorylation in a similar pattern (red signal) with no obvious differences between WT and Hs2st−/− indicating that Hs2st−/− CSB tissue is competent to respond to Fgf17 (Fig. 9B top row). The lack of Fgf17 or pERK signal in the BSA control (Fig 9B, bottom row) confirms Fgf17 antibody specificity and that pERK activation was specifically induced by exogenously applied Fgf17. We performed Sox9/BrdU analysis (exactly as described above for the FGFi experiments - Fig 7) to assess the impact of experimentally introduced Fgf17 on astroglial...
translocation to the midline after 48 hours in culture. The results were dramatic, the side with the Fgf17-bead showed many more Sox9+ (red) cells in the IG region (large arrow on right side of Fig 9C) than the side with the BSA bead (smaller arrow on left side of Fig 9C). Quantification of Sox9+:BrdU+ (yellow) cells (Fig 9D shows higher magnification of IG region) confirmed a significant increase in astroglial translocation to the midline along the rostro-caudal axis on the side exposed to Fgf17 (Fig 9E, compare green (Fgf17) to black (control) lines). An important function of IG glia is to secrete Slit2 and repulsively guide CC axons in the correct trajectory across the midline. At E16.5 Slit2 mRNA is normally expressed in the IG region and in Hs2st−/− embryos the midline Slit2 expression domain is expanded (compare Fig 2C,E to D,F – Slit2 expression domain bracketed). Experimentally introduced Fgf17 is sufficient to phenocopy this aspect of the Hs2st−/− phenotype in our ex vivo assay as the side exposed to the Fgf17 bead has a much larger Slit2 domain than the BSA treated side (compare left (Fgf17) and right (control) bracketed areas in Fig 9F) consistent with precocious translocation of excessive numbers of Slit2+ IG glia.

We conclude that Hs2st−/− CSB tissue is competent to respond to Fgf17 protein and abnormally high levels of Fgf17 protein are sufficient to phenocopy the Hs2st−/− astroglial translocation phenotype consistent with the model presented in Fig 10.

**Hs2st selectively facilitates physical interaction between Fgf17 protein and HS.**

Our in vivo data show that Hs2st suppresses the levels of Fgf17 protein and that this represents a selective interaction between Hs2st mediated 2-O HS sulphation and Fgf17 protein levels in vivo because Hs2st does not suppress the levels of the closely related Fgf8 protein while Hs6st1, which catalyses 6-O HS sulphation, does not suppress Fgf17 protein levels (Chan et al., 2017; Clegg et al., 2014, Fig 8). These in vivo experiments do not however resolve whether differential sulphation has a correspondingly direct selective effect on the physical interaction between HS and Fgf17. In order to test the hypothesis that Hs2st has a selective effect on the binding of Fgf17 protein to HS molecules we turned to a biochemical assay, the ligand and carbohydrate engagement (LACE) assay, that probes physical interaction between HS and FGF proteins by quantifying the ability of endogenous HS in tissue sections to form Fgf:Fgfr:HS complexes with exogenously added Fgf protein and Fgfr ectodomain fused to an Fc tag for immunofluorescent detection (Allen et al., 2001; Chan et al., 2015). We used the Fgf17:Fgfr1 LACE assay to compare the binding of Fgf17 protein to HS in WT, Hs2st−/− and Hs6st1−/− CSB tissue at E14.5 and E16.5 in order to test the hypothesis that Fgf17:HS physical interaction is selectively sensitive to loss of 2-O HS
sulphation in *Hs2st*/*-/- tissue (Fig 11 A-J, O). We used the Fgf8:Fgfr3 LACE assay to compare
the binding of Fgf8 protein to HS in WT and *Hs2st*/*-/- CSB tissue at E14.5 and E16.5 to test the
hypothesis that the Fgf8:HS physical interaction is insensitive to loss of 2-O HS sulphation in
*Hs2st*/*-/- tissue (Fig 11 K-N, P).

In both E14.5 and E16.5 WT tissue the Fgf17:Fgfr1 and Fgf8:Fgfr3 LACE assays
produced a strong LACE signal (Fig 11 A,F,K with higher magnification of boxed areas
enclosing CSB region shown in A’, F’, K’). Control experiments show this LACE signal was
drastically reduced by pre-treating the tissue with heparinitase to digest HS (Fig 11 D,I,M,
with higher magnification of CSB in D’,I’,M’) or omitting Fgf17 or Fgf8 protein from the
assay (Fig 11 E,J,N with higher magnification of CSB in E’,J’,N’). Together these controls
confirm the LACE signal provides a specific readout of the interaction between each FGF
protein and HS molecules. To determine the effect of differential sulphation on the physical
interaction between HS and Fgf8 or Fgf17 we examined how the LACE signal was affected
when the assay was performed on *Hs2st*/*-/- and *Hs6st1*/*-/- tissue. As predicted by our hypothesis
the binding of Fgf17 to HS is selectively sensitive to 2-O HS sulphation as we found that the
Fgf17:Fgfr1 LACE signal was much weaker than WT in *Hs2st*/*-/- tissue (compare B,B’,G,G’ to
A,A’,F,F’) but similar to WT in *Hs6st1*/*-/- tissue (compare C,C’,H,H’ to A,A’,F,F’).
Quantification of Fgf17:Fgfr1 LACE signal intensity in Fig 11 O shows a significant ~4-fold
reduction in *Hs2st*/*-/- (green bar) compared to WT (blue bar) but no significant difference to
WT in *Hs6st1*/*-/- (purple bar). As predicted by our hypothesis that the binding of Fgf8 to HS is
not sensitive to 2-O HS sulphation we found that there was no difference in the Fgf8:Fgfr3
LACE signal between WT and *Hs2st*/*-/- tissue (compare Fig 11 K,K’ to L,L’). Quantification
of Fgf8:Fgfr3 LACE signal intensity in Fig 11 P shows no significant difference between
*Hs2st*/*-/- (green bar) compared to WT (blue bar). These LACE results are summarised
schematically in Fig 11Q which shows that of the five HST genotype and FGF ligand
permutations tested only the Fgf17:HS physical interaction is sensitive to *Hs2st* genotype, as
predicted by the hypothesis that 2-O HS sulphation has a specific effect on the ability of HS
to bind Fgf17.

**Discussion**

Embryonic corpus callosum (CC) development involves multiple cell and molecular
events that ultimately guide callosal axons across the telencephalic midline to connect with
their synaptic targets in the contralateral hemisphere. Three subpopulations of midline
astroglia play pivotal roles in guiding callosal axons across the telencephalic midline. Midline
zipper (MZ) glia facilitate fusion of the cerebral hemispheres and provide a substrate for crossing callosal axons while Slit2⁺ indusium griseum (IG) and glial wedge (GW) astroglia channel crossing axons into the correct path by Robo/Slit mediated chemorepulsion (Bagri et al., 2002; Gobius et al., 2016; Shu and Richards, 2001; Shu et al., 2003). These astroglial populations originate from RGCs born in the VZ of the septal midline and either remain in the VZ at the glial wedge (GW astroglia) or translocate in response to FGF signals, of which Fgf8 appears to be particularly important, to the pial surface of the telencephalic midline (MZ and IG astroglia) (Clegg et al., 2014; Gobius et al., 2016; Moldrich et al., 2010; Smith et al., 2006). Both Slit2⁺ IG and Slit2⁻ MZ astroglia are essential for CC development and both these astroglial populations originate from the septal VZ Zic4 lineage so the lack of an overt CC phenotype in Hs2st_F/F;Zic4_Cre embryos following conditional knockout of Hs2st in the Zic4 lineage indicates that neither MZ or IG astroglial precursors have a cell autonomous requirement for Hs2st to translocate in appropriate numbers. In Hs2st⁺⁻ embryos there is an expansion of the Slit2 expression domain at the CSB pial surface coinciding with increased Sox9⁺ glial cells and this is phenocopied by application of exogenous Fgf17 to Hs2st⁺/+ CSB ex vivo strongly suggesting increased numbers of Slit2⁺ glial cells at the midline reflect excessive GW→IG somal translocation enlarging the IG (current study). We cannot rule out the possibility that disrupted MZ glial translocation also contributes to the Hs2st⁺⁻ phenotype although this would not alter our conclusion that Hs2st plays a non-cell autonomous role in the Zic4-lineage astroglial translocation phenotype. Our model (Fig 10) posits that ectopic Slit2⁺ astroglia at the midline block the transit of CC axons. In principle this could be tested by rescuing the CC axon midline crossing in Hs2st⁺⁻;Slit2⁻⁻ embryos (along similar lines to the Slit2 genetic rescue of the Hs6st1⁺⁻ phenotype we reported in Hs6st1⁺⁻;Slit2⁻⁻ embryos (Conway et al., 2011)). However, in contrast to the fully penetrant (100%) Hs6st1⁺⁻ CC phenotype, the partial penetrance (~50%) of the Hs2st⁺⁻ CC phenotype introduces a confounding factor of distinguishing ‘rescued’ from ‘unaffected’ Hs2st⁺⁻ embryos, a problem that would be compounded if only a proportion of embryos destined to be ‘affected’ were rescued (see (Clegg et al., 2014; Conway et al., 2011)) so a prohibitively large number of animals would be required to demonstrate a statistically significant rescue.

Eliminating HS (Ext1 mutagenesis) compared to 2-O HS sulphation (Hs2st mutagenesis) from the same cell lineages allowed us to distinguish physiological functions generally attributable to HS from those specifically requiring 2-O HS sulphation by comparing the Ext1 and Hs2st phenotypes. We found that while Zic4 lineage cells were unable to support CC development when they lacked HS (Zic4_Cre;Ext1_F/F embryos) there...
was no similar requirement for 2-O HS sulphation in the Zic4 lineage (Zic4<sup>Cre</sup>;Hs2st<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos) indicating that Zic4 lineage cells require HS but that 2-O HS sulphation is dispensable for their contribution to CC development, specifically the ability of astroglial precursors to cell autonomously sense translocation signals. We found that HS and 2-O HS sulphation are both required in the Emx1 lineage (Emx1<sup>CreER</sup>;Ext1<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> and Emx1<sup>CreER</sup>;Hs2st<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos) although the axonal and astroglial phenotypes were not identical. Somewhat counterintuitively, removing HS completely from the Emx1 lineage in Emx1<sup>CreER</sup>;Ext1<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos had a less severe effect on the distribution of GFAP<sup>+</sup> midline glia than preserving HS but blocking its 2-O sulphation, as the accumulation of astroglia at the pial surface of the midline was much more pronounced in Emx1<sup>CreER</sup>;Hs2st<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos.

We speculate that completely removing HS from the Emx1 lineage results in a general destabilisation of FGF protein gradients so mitigating precocious somal translocation by Zic4 lineage astroglial precursors (Chan et al., 2017; Qu et al., 2011; Qu et al., 2012; Shimokawa et al., 2011). The relatively normal midline astroglial organisation in Emx1<sup>CreER</sup>;Ext1<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos poses the question of whether glial disorganisation is a major contributor to their CC agenesis phenotype. In Emx1<sup>CreER</sup>;Hs2st<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos the Probst bundles form right next to the midline, consistent with our hypothesis that ectopic Slit2<sup>+</sup> astroglia at the midline are repelling CC axons from crossing the midline (Conway et al., 2011 & current study). In contrast the Probst bundles in Emx1<sup>CreER</sup>;Ext1<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos form much more lateral to the midline at some distance from the IG indicating CC axons are misrouted at an earlier stage of their navigation than in Emx1<sup>CreER</sup>;Hs2st<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos. HS is required cell autonomously for navigating axons to respond to axon guidance molecules, including Netrin1 and Slit2 (Matsumoto et al., 2007; Piper et al., 2006). A plausible explanation is that in Emx1<sup>CreER</sup>;Ext1<sup>F<sub>Ur/F<sub>Ur</sub>Fl</sub></sup> embryos the Emx1 lineage HS-deficient CC axons cannot respond appropriately to guidance cues that would normally guide them towards the midline and are already misrouted before they come under the influence of the midline astroglia. In contrast Hs2st<sup>−/−</sup> CC axons express HS lacking 2-O sulphation that does not affect their ability to respond to guidance cues (current study) so they reach the midline but are prevented from crossing by the ectopic Slit2<sup>+</sup> glia in the expanded IG.

Biochemical (LACE) data shows that physical interaction between Fgf17 and HS is facilitated by Hs2st (but not Hs6st1) and that Hs2st facilitates physical interaction between HS and Fgf17 (but not Fgf8) suggesting a molecular mechanism underpinning Hs2st selectively suppressing levels of Fgf17 in vivo (Allen and Rapraeger, 2003; Chan et al., 2015; Chan et al., 2017; Clegg et al., 2014), current study). We speculate that Hs2st exerts its
selective effect on Fgf17 protein levels because HS lacking 2-O HS sulphation has reduced affinity for Fgf17 (but not Fgf8) so increasing the half-life of Fgf17 (but not Fgf8) in the ECM by selectively reducing the rate that Fgf17 protein is cleared by HS-mediated receptor mediated endocytosis of canonical FGFs while leaving Fgf8 unaffected (Yu et al., 2009). Our conditional mutagenesis experiments clearly demonstrate there is no cell autonomous requirement for Hs2st in astroglial precursor translocation in Emx1CreER;Hs2stFl/Fl embryos, however the reduced efficiency of HS:Fgf17:Fgfr1 complex formation in the LACE assay implies that Hs2st might also play a cell-autonomous role in the response to Fgf17 protein. We speculate that even if Hs2st+/− astroglial precursors are less sensitive to Fgf17 than their wild-type counterparts their translocation to the midline is primarily driven by Fgf8 so is not significantly affected in Zic4Cre;Hs2stFl/Fl embryos. A putative reduced sensitivity of Hs2st+/− astroglial precursor cells to Fgf17 also begs the question of how elevated Fgf17 could trigger precocious glial translocation in Hs2st+/− embryos. The Fgf17 bead assay experiment shows that Hs2st+/− cells retain competence to respond to Fgf17 by phosphorylating ERK and LACE data shows that HS devoid of 2-O HS sulphation still interacts with Fgf17 albeit with reduced efficiency. The explanation that best fits our experimental data, therefore, is that increased Fgf17 protein levels in Hs2st+/− embryos overrides any reduction in competency of Hs2st+/− cells to respond to Fgf17 protein and the net effect is elevated FGF/ERK signalling and consequent precocious astroglial translocation.

This study makes two major novel contributions to our understanding of the cell and molecular roles of differential HS sulphation in the regulation of forebrain development. First that a primary cellular role of 2-O HS sulphation in vivo is not to modulate the competence of astroglial precursor cells to respond to translocation signals by a cell autonomous mechanism (as would be predicted by the classic role for HS in modulating the formation of the FGF:FGFR:HS receptor complex on the surface of responding cells) but instead to regulate the supply of translocation signals to astroglial precursors by a non-cell autonomous mechanism. Second that the interaction between 2-O sulphated HS and Fgf17 protein is selective because it does not apply to the closely related Fgf8 protein or to 6-O HS sulphation catalysed by Hs6st1. The most parsimonious explanation linking these cell and molecular events is that higher than normal levels of Fgf17 protein at the CSB of Hs2st+/− embryos causes the precocious astroglial precursor translocation phenotype and subsequent misrouting of CC axons (Fig 10). Our rescue of the Hs2st+/− precocious astroglial precursor translocation phenotype in vivo by generic pharmacological inhibition of FGF signalling with SU5402 directly supports the hypothesis that hyperactive FGF/ERK signalling causes the phenotype.
Given the well known role of FGF/ERK signalling in triggering astroglial precursor translocation to the IG, our findings that exogenously applied Fgf17 protein is sufficient to phenocopy the $Hs2st^{+/−}$ astroglial precursor translocation phenotype, and that $Hs2st^{+/−}$ CSB cells activate ERK in response to Fgf17 protein, it is extremely unlikely that increased Fgf17 protein levels in vivo wouldn’t result in ERK hyperactivation and consequent precocious astroglial precursor translocation in $Hs2st^{+/−}$ embryos. However, the current study does not provide formal proof that the elevated levels of Fgf17 protein are solely responsible for the FGF/ERK hyperactivation or precocious astroglial precursor translocation phenotypes in $Hs2st^{+/−}$ embryos and we were unable to design an experiment that could further discriminate between the functions of Fgf17 and Fgf8 and directly test functional selectivity of Hs2st for Fgf17 in this context. We considered employing a classic rescue experiment strategy by genetically reducing Fgf17 dosage in $Hs2st^{+/−}$ embryos (‘Fgf17$^{−/−}$;Hs2st$^{+/−}$ rescue) but on balance elected not to because at best it would provide equivocal evidence either for or against the hypothesis that 2-O sulphated HS interacts selectively with Fgf17 protein. FGF/ERK hyperactivation caused by overexpression of a particular FGF protein can be rescued by any experimental manipulation that restores ERK signalling to normal levels and not uniquely by restoring the levels of the FGF protein that underpins the phenotype. Specifically, reducing Fgf17 dosage could elicit a rescue of ERK hyperactivation and collateral phenotypes at the $Hs2st^{+/−}$ CSB by reducing FGF/ERK signalling output whether or not abnormally high Fgf17 bioavailability was the primary cause. Analogously we interpret rescue of the $Hs6st1^{+/−}$ precocious astroglial precursor translocation phenotype in $Hs6st1^{−/−}$;Fgf8neo/neo embryos as evidence that $Hs6st1$ normally acts to keep FGF/ERK signalling in check rather than as evidence for a selective genetic interaction between Fgf8 and $Hs6st1$ (Clegg et al., 2014). Conversely, failure to rescue the $Hs2st^{+/−}$ phenotype in Fgf17$^{−/−}$;Hs2st$^{+/−}$ embryos (or using other methods to reduce Fgf17 protein levels or functionality) would not falsify the hypothesis that increased Fgf17 bioavailability caused the $Hs2st^{+/−}$ phenotype because there are several alternative explanations. When we employed a similar strategy in a similar context to rescue the $Hs6st1^{+/−}$ astroglial precursor precocious translocation phenotype by genetically reducing Fgf8 dosage the rescue was only successful in a minority of isogenic $Hs6st1^{−/−}$;Fgf8neo/neo embryos and a likely explanation is that compensatory mechanisms act when Fgf gene dosage is manipulated (Clegg et al., 2014). Such compensation will generate false negative results making it unsafe to interpret unrescued Fgf17$^{−/−}$;Hs2st$^{+/−}$ embryos as falsifying the hypothesis that the phenotype is underpinned by excess Fgf17 protein. There are additional technical confounds that could lead to false negatives because a rescue likely
requires precise restoration of normal Fgf17 protein levels (so no rescue could reflect technical failure to restore Fgf17 protein levels to normal) and in any case the CC phenotype of Fgf17−/− embryos has not been thoroughly characterised so Hs2st−/−;Fgf17−/− phenotypes may well be problematic to interpret (Cholfin and Rubenstein, 2007, 2008). In addition to not being decisive for or against selectivity we note that demonstrating genetic interaction between Hs2st and Fgf17 would not provide insight into whether the interaction was molecularly direct or not, in contrast to biochemical LACE data we present in the current study.

The closely related ‘Fgf8 subfamily’ members Fgf17 and Fgf8 are both transcribed by cells in the CSB region yet have different roles in forebrain development with available evidence, while not ruling out a role for Fgf17, suggesting that Fgf8 is the primary driver of astroglial precursor translocation required for CC development (Cholfin and Rubenstein, 2007, 2008; Gobius et al., 2016; Moldrich et al., 2010; Toyoda et al., 2011). The independent suppression of Fgf17 and Fgf8 protein levels by HS modified by Hs2st and Hs6st1 respectively may have facilitated the evolution of this system by providing a mechanism to tilt the Fgf17:Fgf8 protein balance to give Fgf8 the more dominant role in regulating astroglial precursor translocation (Chan et al., 2017; Clegg et al., 2014, Current study). In this sense there are parallels to other negative regulatory strategies, for example micro-RNAs that function by protecting cells from the expression of particular proteins that would be detrimental if expressed.

**Figure Legends:**

**Figure 1.** Hs2st protein is expressed in the cerebral cortex and the septum during CC formation. (A) Immunohistochemistry for Hs2st at E14.5 (B-D) Higher magnification shows punctate subcellular Hs2st expression (inset, B) Hs2st protein is expressed at the CSB (B), the VZ of the cortex (C) and the cortical plate (D), (E) Immunohistochemistry for Hs2st at E18.5 (F-M) Hs2st protein is expressed in the IG (F), the GW (G), the septum (H), and the ventral telencephalon (I). Within the cortex Hs2st is expressed at the ventricular zone (J), the intermediate zone (K), Hs2st is not strongly expressed by the middle layers of the cortex (L), but is expressed by the deeper layers (M). (N-R) Hs2st antibody specificity. The Hs2st antibody produces signal in the GW (J,L), which is lost in Hs2st−/− embryos (K,M). Western-blot performed on protein extracted from whole telencephalon using Hs2st antibody reveals the predicted ~42kDa band in WT extracts, which is lost in Hs2st−/− extracts (N). B-D are
higher magnification images of boxed regions indicated in A. F-M are higher magnification images of boxed regions indicated in E. L and M are higher magnification images of boxed regions in J and K respectively. Insets in B, F, P and Q are higher magnification images of boxed regions. Scale bars: 500μm in A, 50μm in B-I, L, M; 100μm in J.K.

**Figure 2.** Slit2 expression at the CSB of WT and Hs2st-/ embryos at E16.5. (A, C, E) *In-situ* hybridisation for Slit2 in WT embryos at 3 rostro-caudal positions labelling the GW and IG. (B, D, F) *In situ* hybridisation for Slit2 in Hs2st-/ embryos at equivalent positions to A, C and E respectively showing an expanded IG. Scale bar: 100μm in all panels.

**Figure 3.** Emx1 and Zic4 lineage contribution at the CSB. (A) Zic4<sup>Cre</sup> allele combined with a lox-stop GFP reporter has been used to label cell populations at E18.5. Zic4<sup>Cre</sup> labels cells of the septum. (B, C, D, E) Zic4<sup>Cre</sup> labels cells ventral to the CSB (dashed line, B,C) including Sox9 expressing cells (arrowheads, C) but is not expressed by Sox9 expressing cells dorsal to the CSB (arrows, C). Zic4<sup>Cre</sup> is expressed by IG glial cells (arrowheads, D) but not by surrounding cells. Zic4<sup>Cre</sup> is expressed by MZ glial cells (arrowheads, E). (F) Schematic of the Zic4<sup>Cre</sup> expressing cell lineage. (G) Emx1<sup>CreER</sup> allele combined with a lox-stop GFP reporter has been used to label cell populations at E18.5. Emx1<sup>CreER</sup> labels cells of the cortex. (H, I, J, K) Emx1<sup>CreER</sup> labels cells dorsal to the CSB (dashed line, H,I) including Sox9 expressing cells (arrowheads, I) but is not expressed by Sox9 expressing cells ventral to the CSB (arrows, I). Emx1<sup>CreER</sup> is not expressed by IG glial cells (arrows, J). Emx1<sup>CreER</sup> is not expressed by MZ glial cells (arrows, K). (L) Schematic of the Emx1 expressing cell lineage. No phenotype was detected in Hs2st<sup>+/+</sup> Zic4<sup>Cre</sup> or Hs2st<sup>+/+</sup> Emx1<sup>CreER</sup> embryos (n=5 for each genotype). C, D and E are higher magnification images of the indicated regions in B. I, J and K are higher magnification images of the indicated regions in H. Scale bars: 500μm in A,G; 200μm in B and H; 50μm in C-E and I-K.

**Figure 4.** HS expression is required within both Emx1 and Zic4 lineage cells for CC formation. (A-I) Immunofluorescence for L1 (red) at E18.5 labels the CC while GFAP (green) labels glia. In control embryos the U-shaped CC has formed and is flanked by glia at the IG and GW (A, D, G). In Ext1<sup>fl/fl</sup> Emx1<sup>CreER</sup> embryos CC axons do not cross the midline while glia at the IG and GW appear largely unaffected (B, E, H). In Ext1<sup>fl/fl</sup> Zic4<sup>Cre</sup> embryos CC axon do not cross the midline while glia appear depleted at the IG and form abnormal bundles at the GW (C, F, I). (J-O) FGFR1/ FGF2 ligand and carbohydrate engagement
(LACE) assay is used to detect the presence of HS. In control embryos LACE signal can be seen throughout the telencephalon, and is of similar intensity within both the cortex and the septum (J, M). In Ext1\textsuperscript{fl/fl} Emx1\textsuperscript{creER} embryos LACE signal is significantly reduced within the cortex (K, N). In Ext1\textsuperscript{fl/fl} Zic4\textsuperscript{cre} embryos LACE signal is significantly reduced within the septum (L, O). D-I are higher magnification images of the indicated boxed regions in A-C, J-L are higher magnification images of the boxed region in G-I respectively. Scale Bars: 200μm in A-C and G-I; 100μm in D-F and J-L.

Figure 5. Hs2st expression is required within Emx1 lineage cells but not Zic4 lineage cells for CC formation. (A-C) Immunofluorescence for L1 and GFAP at E18.5. In control embryos the U-shaped CC has formed and the IG can be observed above the CC (A). In Hs2st\textsuperscript{fl/fl} Zic4\textsuperscript{cre} embryos the CC and IG form normally (B). In around half of Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} embryos the CC fails to form, IG glia also extend ventrally (asterisks, C). In the remaining Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} embryos the CC forms normally (C'). (D-I) Immunofluorescence for Sox9 labels progenitor cells at the ventricular zone and mature glia at the IG, GFP labels cells in which cre is active. (D, G) In control (Hs2st\textsuperscript{+/+};Emx1\textsuperscript{creER}) embryos IG glia do not express GFP. (E, H) In Hs2st\textsuperscript{fl/fl};Zic4\textsuperscript{cre} embryos IG glia do express GFP and adopt their normal position. (F, I) In Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} embryos GFP is expressed by cortical neurons and axons but not by abnormally positioned IG glia. (J-L) Immunohistochemistry for Hs2st shows expression of Hs2st in the IG. (J) in control embryos punctate Hs2st staining can be seen within IG cells. In Hs2st\textsuperscript{fl/fl};Zic4\textsuperscript{cre} embryos Hs2st is not expressed by IG glia (K). In Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} embryos Hs2st is expressed by displaced glial cells (L). Hs2st immunohistochemistry in J-L was performed on adjacent tissue sections to those in D-I. (M) Quantification of Sox9 expressing cell number at the IG in control (blue bar, n=4 embryos, 2 Hs2st\textsuperscript{+/+};Zic4\textsuperscript{cre} + 2 Hs2st\textsuperscript{+/+};Emx1\textsuperscript{creER}), affected Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} (orange bar, n=4 embryos), and Hs2st\textsuperscript{fl/fl};Zic4\textsuperscript{cre} (purple bar, n=3 embryos). Sox9\textsuperscript{*} numbers are significantly increased compared to control in Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} embryos (* indicates p<0.05 on graph), (F(2, 7) = 42.16, p = 0.00013, ANOVA), post-hoc t-tests: control vs Hs2st\textsuperscript{fl/fl};Emx1\textsuperscript{creER} (t(4) = -8.08, p = 0.0013, t-test); & control vs Hs2st\textsuperscript{fl/fl};Zic4\textsuperscript{cre} (t(5) = 0.92, p = 0.40, t-test). Boxed regions in G-I are higher magnification images of boxed regions in D-F respectively. Insets in J, K and L are higher magnification images of boxed region shown on each image. Scale bars: 200μm in A-F; 100μm 50μm in G-I and J-L.
Figure 6: Hs2st is not required by CC axons in order to cross the telencephalic midline. (A, D) After homotypic transplantation of E17.5 cortical explants from GFP+ control tissue into the cortex of GFP+ control brain slices GFP+ CC axons are able to project across the midline (arrows, D). (B, E) After transplantation of GFP+ Hs2st−/− cortical explants into GFP+ control brain slices GFP+ CC axons are able to project across the midline (arrows, E). (C, F) After transplantation of cortical explants from GFP+ control tissue into the cortex of GFP− Hs2st−/− brain slices GFP+ CC axons are unable to project across the midline and invade the septum. Arrowheads indicate axons navigating into the septum in all conditions. (G-I) Schematic of transplant experiments shown in A-C. D-F are higher magnification images of the boxed region in A-C respectively. Scale bars: 200μm in all panels.

Figure 7: Hyperactive Fgf signalling causes precocious glia translocation in Hs2st−/− CSB. (A) Experimental outline of Hs2st−/− phenotypic rescue experiment. Pregnant females were injected at E14.5 with a BrdU pulse and CSB slices collected after 1 hour and cultured for 48hrs (B-I) WT or Hs2st−/− CSB slices were cultured in the presence of either SU5402 (FGFi) or DMSO (untreated vehicle control). (B, D, F, H) pErk immunohistochemistry shows that FGFi treatment reduces Fgf/ERK signalling. (C,E,G,I) Immunofluorescence for BrdU and Sox9 in WT (C,E) and Hs2st−/− (G,I) slices treated with FGFi (E,I) or untreated (C,G), the curved dotted line demarcates the basal edge of the VZ, arrows in C,G point to accumulations of BrdU/Sox9+ cells at the midline (arrow size corresponds to cell number) with higher magnification insets showing Sox9/BrdU+ (yellow) double labelled cells in IG region. (J) Quantification of Sox9/BrdU+ double labelled cells in WT or Hs2st−/− CSB slice cultures treated with FGFi or untreated (n=3 embryos for each condition). For both genotypes FGFi treatment significantly reduces the number of Sox9/BrdU+ cells that exit the VZ and moved towards the IG at one or more rostro-caudal position (significant differences due to FGFi treatment within each genotype indicated on graph as **p<0.05 ***p<0.001). (F(3, 32) = 31.00, p = 0.0000000014, Two-way ANOVA) followed by t-test with Sidak’s correction for multiple comparisons at each positon along the rostro-caudal axis. WT FGFi vs WT untreated: position 1 (t(16) = 1.67, p = 0.24, t-test); position 2 (t(16) = 2.37, p = 0.11, t-test); position 3 (t(16) = 2.25, p = 0.15, t-test); & position 4 (t(16) = 2.81, p = 0.050, t-test). Hs2st−/− FGFi vs Hs2st−/− untreated: position 1 (t(16) = 2.38, p = 0.11, t-test); position 2 (t(16) = 2.83, p = 0.048, t-test); position 3 (t(16) = 3.05, p = 0.030, t-test); & position 4 (t(16) = 4.60, p = 0.0012, t-test). Scale bars: 100μm B-I.
Figure 8. Expression of Fgf17 during CSB development in WT and Hs2st<sup>-/-</sup> and Hs6st1<sup>-/-</sup> embryos. (A-C) Fgf17 protein and mRNA expression at the E12.5 CSB of WT and Hs2st<sup>-/-</sup> embryos. Fgf17 protein is expressed across the CSB in both WT and Hs2st<sup>-/-</sup> embryos, with no obvious change in intensity or domain of expression. Fgf17 mRNA expression overlaps well with the protein expression domain and is similar between WT and Hs2st<sup>-/-</sup>. (D-F) Fgf17 protein and mRNA expression at the E14.5 CSB of WT and Hs2st<sup>-/-</sup> embryos. Fgf17 protein is expressed at low levels at the CSB of WT embryos. In Hs2st<sup>-/-</sup> embryos, the protein expression domain expands across the CSB (asterisks in E2). Fgf17 mRNA is unchanged at the CSB between WT and Hs2st<sup>-/-</sup> embryos. (G-I) Fgf17 protein and mRNA expression at the E16.5 CSB of WT and Hs2st<sup>-/-</sup> embryos. Fgf17 protein is expressed at low levels at the CSB of WT embryos (H1). In Hs2st<sup>-/-</sup> embryos, the protein expression domain expands (asterisks, H2). There is a concurrent increase in Fgf17 mRNA (asterisk, I2). (J-O) Fgf17 mRNA (J, K<sub>1,2,3</sub>) and Fgf17 protein (M, N<sub>1,2,3</sub>) expression (red) relative to GFP<sup>+</sup> Zic4 lineage cells (indicated with white arrows) at the GW (K<sub>1,2,3</sub>, N<sub>1,2,3</sub>) and IG (L<sub>1,2,3</sub>, O<sub>1,2,3</sub>) of WT E14.5 embryos. (P) E14.5 expression of Hs2st by LacZ staining. Hs2st is expressed most highly at the VZ, with decreasing expression towards the pial surface. (Q-V) Fgf17 protein and mRNA expression at the E14.5 CSB of WT and Hs6st1<sup>-/-</sup> embryos. Fgf17 protein is expressed at low levels at the CSB of WT embryos (Q1,R1). In Hs6st1<sup>-/-</sup> embryos, the protein expression domain is similar to WT (Q2,R2). Fgf17 mRNA expression is unchanged between WT and Hs6st1<sup>-/-</sup> embryos (S1, S2). (T-V) Fgf17 protein and mRNA expression at the E16.5 CSB of WT and Hs6st1<sup>-/-</sup> embryos. Fgf17 protein is expressed at very low levels at the CSB of both WT (T1, U1) and Hs6st1<sup>-/-</sup> (T2, U2) embryos. Fgf17 mRNA expression is unchanged between WT (V1) and Hs6st1<sup>-/-</sup> (V2) embryos. (W) Quantification of Fgf17 immunofluorescence signal at CSB in WT (blue bar, n=3 embryos), Hs2st<sup>-/-</sup> (green bar, n=3 embryos) and Hs6st1<sup>-/-</sup> (purple bar, n=3 embryos). Fgf17 protein level is significantly increased compared to WT in Hs2st<sup>-/-</sup> embryos (* indicates p<0.05 on graph). (F(2, 9) = 13.83, p = 0.0018, ANOVA), post-hoc t-tests: WT vs Hs2st<sup>-/-</sup> (t(4) = -4.22, p = 0.014, t-test); & WT vs Hs6st1<sup>-/-</sup> (t(6) = -0.98, p = 0.36, t-test). Boxed areas in A,D,G,Q,T shown at higher magnification in B,E,H,R,U respectively. Scale bars: 200μm in A,D,G,Q,T; 100μm in B,C,E,F,H,I,R,S,U,V,J,M,P; 10μm in K,L,N,O.
Figure 9. Fgf17-bead experiment. (A) Experimental outline of Fgf17 protein bead experiment in WT embryos. Pregnant females were injected at E14.5 with a BrdU pulse and CSB slices collected. One Fgf17 and one BSA bead were added to each side of the midline. (B) Fgf17 protein and pErk after 2 hours in culture. In both WT and Hs2st−/− CSB slices, Fgf17 and pErk are seen in tissue surrounding the Fgf17 bead. Staining for either is absent around the BSA bead (demarcated by dotted circle). (C) Immunofluorescence for BrdU and Sox9 was performed on slices after 48 hours in culture, curved dotted lines indicate the basal edge of the VZ and straight dotted line indicates the midline. D shows a higher power of the arrowed regions in C. (E) Quantification of Sox9+/BrdU+ double labelled cells in CSB slice cultures with Fgf17 or BSA bead. The Fgf17 bead significantly increased the number of Sox9+/BrdU+ cells that exit the VZ and moved towards the IG (significant differences indicated on graph as **p<0.05 ***p<0.001) at the four caudal-most positions (n=5 embryos). (F(1, 48) = 65.63, p = 0.0000000000155, Two-way ANOVA) followed by t-test with Sidak’s correction for multiple comparisons for Fgf17-bead vs BSA-bead at each rostral-caudal position: position 1 (t(48) = 1.45, p = 0.63, t-test); position 2 (t(48) = 2.36, p = 0.13, t-test); position 3 (t(48) = 3.65, p = 0.0039, t-test); position 4 (t(48) = 4.47, p = 0.0003, t-test); position 5 (t(48) = 4.03, p = 0.0012, t-test); & position 6 (t(48) = 3.89, p = 0.0018, t-test). (F) Slit2 expression in slices cultured with Fgf17 and BSA beads. Scale bars: 100μm.

Figure 10 Model. Hs2st expressed in Emx1 lineage cells catalyses 2-O HS sulphation (2-O HS) that in turn supresses levels of Fgf17 protein, but not Fgf8 protein, by an unknown mechanism at the CSB. Zic4 lineage astroglial precursors respond to Fgf8 and Fgf17 protein by activating FGF/ERK signalling and translocating (black arrows) to the midline. This generates appropriate positioning of Slit2+ astroglia to guide corpus callosum axons across the midline. Loss of 2-O HS from the Emx1 lineage selectively de-supresses Fgf17 protein levels while leaving Fgf8 protein unaffected. This causes hyperactive FGF/ERK signalling and more Zic4 lineage astroglial precursors translocate than normal with consequent blocking of corpus callosum axon midline crossing by the ectopic midline Slit2+ astroglia. Zic4 lineage astroglial precursor cells do not need to express 2-O HS in order to respond to FGF signalling proteins and translocate to the midline.
Figure 11 Ligand and carbohydrate engagement (LACE) assay for FGF:HS interactions. (A-J, O) FGFR1/FGF17 LACE experiments on (A-E) E14.5 and (F-J) E16.5 telencephalic coronal sections through the CSB. (A, F) WT, (B, G) Hs2st⁻/⁻, (C, H) Hs6st1⁻/⁻ (D, I) WT sections pretreated with Heparinase to digest HS, (E, J) WT sections with FGF17 omitted from the LACE assay. (O) Quantification of FGF17/FGFR1 LACE signal in WT (blue bar, n=9 embryos), Hs2st⁻/⁻ (green bar, n=5 embryos), and Hs6st1⁻/⁻ (purple bar, n=4 embryos), showing a significant decrease (* indicates p<0.05 on graph) in Hs2st⁻/⁻ embryos, (F(2, 15) = 8.62, p = 0.0032, ANOVA), followed by post-hoc t test: WT vs Hs2st⁻/⁻ (t(9) = 6.11, p = 0.014, t-test); & WT vs Hs6st1⁻/⁻ (t(5) = 0.63, p = 0.56, t-test) (K-N, P) FGFR3/FGF8 LACE experiments on E14.5 telencephalic coronal sections through the CSB. (K) WT, (L) Hs2st⁻/⁻, (M) WT sections pretreated with Heparinase to digest HS, (N) WT sections with FGF8 omitted from the LACE assay. (P) Quantification of FGF8/FGFR3 LACE signal in WT (blue bar, n=3 embryos), Hs2st⁻/⁻ (green bar, n=3 embryos) shows no significant difference (t(3) = 0.29, p = 0.76, t-test). Numbers of embryos of each genotype analysed indicated under bars. (Q) Summary diagram. FGFR1/FGF17/HS complex formation is equally supported by WT and Hs6st1⁻/⁻ HS but less so by Hs2st⁻/⁻ HS while FGFR3/FGF8/HS complex formation is equally supported by WT and Hs2st⁻/⁻ HS showing that FGF17:HS physical molecular interaction is selectively dependent on 2-O HS sulphation. Higher magnification showing the CSB region boxed in A-N are shown in A’-N’ (note the DAPI channel is not shown in the higher magnification images to improve visualisation of the LACE signal). Scale bars 200μm.

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E18.5

| Control (Hs2st<tr>Emx1Cre<sup>ER</sup>) | Hs2st<sup>tr</sup>; Zic4Cre | Hs2st<sup>tr</sup>; Emx1Cre<sup>ER</sup> |
|-------------------------------------|-----------------------------|---------------------------------|
| ![Image A](imageA.png)              | ![Image B](imageB.png)      | ![Image C](imageC.png)          |
| ![Image D](imageD.png)              | ![Image E](imageE.png)      | ![Image F](imageF.png)          |
| ![Image G](imageG.png)              | ![Image H](imageH.png)      | ![Image I](imageI.png)          |
| ![Image J](imageJ.png)              | ![Image K](imageK.png)      | ![Image L](imageL.png)          |

**M**

Sox9 Cells at the IG

![Graph](imageM.png)
A  Inhibiting Fgfr1 signalling

E14.5 1hr BrdU pulse

+ DMSO or FGF1 [SU5402]

| pErk | Sox9  | BrdU |
|------|------|------|
| B    |      |      |
| D    |      |      |
| F    |      |      |
| H    |      |      |

J

No. Sox9/BrdU+ cells

Rostral-caudal position

** Hs2st<sup>−/−</sup>

* WT

WT + FGF1

Hs2st<sup>−/−</sup> + FGF1
|        | E12.5 |       | E14.5 |       | E16.5 |       |
|--------|-------|-------|-------|-------|-------|-------|
| WT     |       |       | WT    |       | WT    |       |
| H\text{\textsuperscript{s2t}}\textsuperscript{+} |       |       |       |       |       |       |

|        | A\textsubscript{1} | Cortex | A\textsubscript{2} | Cortex | D\textsubscript{1} | Cortex | D\textsubscript{2} | Cortex | G\textsubscript{1} | Cortex | G\textsubscript{2} | Cortex |
|--------|-------------------|--------|-------------------|--------|-------------------|--------|-------------------|--------|-------------------|--------|-------------------|--------|
|        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        |
|        | B\textsubscript{1} | Septum | B\textsubscript{2} | Septum | E\textsubscript{1} | Septum | E\textsubscript{2} | Septum | H\textsubscript{1} | Septum | H\textsubscript{2} | Septum |
|        | Fg\text{f17} mRNA |        | Fg\text{f17} mRNA |        | Fg\text{f17} mRNA |        | Fg\text{f17} mRNA |        | Fg\text{f17} mRNA |        | Fg\text{f17} mRNA |        |
|        | C\textsubscript{1} |        | C\textsubscript{2} |        | F\textsubscript{1} |        | F\textsubscript{2} |        | I\textsubscript{1} |        | I\textsubscript{2} |        |
|        |                  |        |                  |        |                  |        |                  |        |                  |        |                  |        |

| Zic\text{\textsuperscript{4}}\textsuperscript{\textdagger} | Fg\text{f17} | GFP | Merge | Fg\text{f17} | GFP | Merge |
|-----------------|-------------|-----|-------|-------------|-----|-------|
| Midline         | GW          | IG  |       | IG          |     |       |
|                  |                 |     |       |                 |     |       |

|        | E14.5 |       | E16.5 |       |
|--------|-------|-------|-------|-------|
| WT     |       |       | WT    |       |
| H\text{\textsuperscript{s6t1}}\textsuperscript{-} |       |       |       |       |

|        | Q\textsubscript{1} | Cortex | Q\textsubscript{2} | Cortex | T\textsubscript{1} | Cortex | T\textsubscript{2} | Cortex |
|--------|-------------------|--------|-------------------|--------|-------------------|--------|-------------------|--------|
|        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        | DAPI/Fg\text{f17} |        |
|        | S\textsubscript{1} |        | S\textsubscript{2} |        | V\textsubscript{1} |        | V\textsubscript{2} |        |
| W     | FGF17 Expression  |        |                  |        |                  |        |                  |        |
|       |                   |        |                  |        |                  |        |                  |        |

* denotes significant difference.
Ectopic expression of Fgf17

E14.5 1hr BrdU pulse

B

| pErk | Fgf17 | after 2hrs |
|------|-------|-----------|
| WT   |       |           |
| Hs2st^{-/-} |   |           |

BSA

Sox9 BrdU

E

WT + Fgf17

No. Sox9/BrdU+ cells

Rostral-caudal position

WT + beads

Hs2st^{-/-}

BSA

Fgf17

Slt2
$Hs2st^{+/+}$

$\downarrow$ 

2-O HS

GW

$Fgf17$

$Fgf8$

IG

$Zic4$ lineage glial cells

2-O HS 2-O sulphation of $Emx1$ lineage cells

Callosal axons

$Hs2st^{-/-}$
