RESEARCH REPORT

2- and 6-O-sulfated proteoglycans have distinct and complementary roles in cranial axon guidance and motor neuron migration

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ABSTRACT

The correct migration and axon extension of neurons in the developing nervous system is essential for the appropriate wiring and function of neural networks. Here, we report that O-sulfotransferases, a class of enzymes that modify heparan sulfate proteoglycans (HSPGs), are essential to regulate neuronal migration and axon development. We show that the 6-O-sulfotransferases HS6ST1 and HS6ST2 are essential for cranial axon patterning, whilst the 2-O-sulfotransferase HS2ST (also known as HS2ST1) is important to regulate the migration of facial branchiomotor (FBM) neurons in the hindbrain. We have also investigated how HS2ST interacts with other signals in the hindbrain and show that fibroblast growth factor (FGF) signalling regulates FBM neuron migration in an HS2ST-dependent manner.

KEY WORDS: Fibroblast growth factor (FGF), Vascular endothelial growth factor (VEGF), Heparan sulfate proteoglycan (HSPG), Sulfotransferase, Neuron migration, Axon guidance

INTRODUCTION

Various developmental processes require heparan sulfate proteoglycans (HSPGs), extracellular matrix proteins with covalently linked polysaccharide side chains that are polymerised by exotin enzymes and further modified by sulfation, epimerisation or deacetylation to generate vast structural and functional heterogeneity (Kreuger and Kjellen, 2012). Two families of modifying enzymes, the 2- and 6-O-sulfotransferases, have previously been shown to regulate axon guidance in the Xenopus and mouse brain (Irie et al., 2002; Pratt et al., 2006; Clegg et al., 2014). Both enzymes also control neuronal migration in Caenorhabditis elegans (Kinnunen et al., 2005). However, it is not known whether these enzymes regulate neuronal migration in the vertebrate brain.

The proper positioning of neurons in the developing brain and the correct extension of their neurites to suitable targets are fundamental processes for the appropriate wiring and therefore function of the nervous system. The facial (VIIth) and trigeminal (Vth) cranial nerves are widely used models to define molecular mechanisms of axon guidance (Chandrasekhar, 2004; Wanner et al., 2013). Whilst the trigeminal motor neurons arise in hindbrain rhombomere 2 (r2), the facial branchiomotor (FBM) neurons arise in r4. Both types of neurons extend their axons dorsally out of the hindbrain into the branchial arches in stereotypical patterns that can be visualised by whole-mount immunostaining. Mouse FBM neurons are useful to study neuronal migration, because ISL1 labelling can be used to observe their cell bodies as they migrate caudally from r4 through r5 before turning ventrally to reach their final position in r6 (e.g. Schwarz et al., 2004). Several molecular signals cooperatively control FBM neuron migration (Chandrasekhar, 2004; Wanner et al., 2013), for example, the secreted extracellular matrix protein reelin and components of the WNT planar cell polarity (PCP) pathway (Rossel et al., 2005; Vivancos et al., 2009; Qu et al., 2010). The vascular endothelial growth factor (VEGF-A; short, VEGF) also guides FBM neuron migration through its receptor neuropilin 1 (NRP1); interestingly, NRP1 also serves as a receptor for repulsive sema3A signals during FBM axon guidance (Taniguchi et al., 1997; Schwarz et al., 2004).

O-sulfotransferases have previously been implicated in VEGF, fibroblast growth factor (FGF) and WNT signalling (e.g. Ai et al., 2003; Kamimura et al., 2006; Mitsu et al., 2006). The 6-O-sulfotransferases HS6ST1 and HS6ST2 promote the formation of VEGF/VEGFR and FGF/FGFR signalling complexes in vascular endothelial cells (Ferreras et al., 2012). HS6ST2 is also required for VEGF signalling during zebrafish vascular development (Chen et al., 2005). Moreover, FGFs interact with O-sulfated HSPGs during lacrimal gland and tracheal development (Kamimura et al., 2006; Qu et al., 2011). WNT signalling is regulated by 2-O-sulfotransferase (HS2ST, also known as HS2ST1) during zebrafish epiboly and by HS6STs for muscle development (Bink et al., 2003; Cadwalader et al., 2012), suggesting that the role of O-sulfated HSPGs is context-specific.

Here, we show that mice lacking HS6ST1 and HS6ST2 have defective axon extension of specific branches of the Vth and VIIth cranial nerves, but normal FBM neuron migration. Conversely, mice lacking HS2ST showed normal cranial axon patterning, but had FBM neuron migration defects similar to those seen in mice lacking VEGF signalling through NRP1. Surprisingly, however, HS2ST was not required for VEGF signalling in these neurons. Instead, HS2ST enabled FGF-mediated FBM neuron migration in hindbrain explants. Moreover, the expression of Erm, a known FGF target, was altered in these mutants. Thus, our study has revealed a role for HSPG-mediated FGF signalling in neuronal migration and demonstrated distinct and complementary roles for 2- and 6-O-sulfotransferases in cranial nerve development.
RESULTS AND DISCUSSION

Hs6st1 and Hs6st2 cooperate in cranial axon guidance, but are dispensable for FBM neuron migration

To examine whether HSPGs regulate FBM neuron migration, we used an ex vivo hindbrain culture assay (Fig. 1A). In this model, FBM neurons migrate rostrally from their site of origin in r4 towards r6, similar to the migration they undergo in vivo (Tillo et al., 2014). The inclusion of heparitinase in the culture medium to remove heparan sulfate side chains from HSPGs (Linhardt et al., 1990) prevented FBM neuron migration beyond r5 (Fig. 1B). This observation suggested that HSPGs are important for FBM neuron migration. As HS6ST2 is also required for VEGF signalling during zebrafish vascular development, we examined the expression of 6-O-sulfotransferases during FBM migration. In situ hybridisation (ISH) at 12.5 days post coitum (dpc) showed that Hs6st3 was not obviously expressed in the hindbrain at this stage (data not shown), but Hs6st1 and Hs6st2 were expressed in the r5/6-derived hindbrain territories, through which the Isl1-expressing FBM neurons migrate (Fig. 1C). However, Hs6st1−/− and Hs6st2−/− single mutants had normal FBM neuron migration, and two out of five double mutant Hs6st1−/− Hs6st2−/− hindbrains examined showed only minor displacement of some neurons (Fig. 1D). TUJ1 whole-mount immunostaining of Hs6st1−/− and Hs6st2−/− heads showed normal guidance of facial nerve axons, including those in the facial branchiomotor nerve (VIIbm), the chorda tympani (VIIct) and the greater superficial petrosal nerve (VIIgspn) (Fig. 1E). Trigeminal axons also seemed normal in these single mutants, including those of the mandibular (Vmd), maxillary (Vmx) and ophthalmic (Vop) nerves. However, the VIIbm, VIIct and VIIgspn and Vmx nerves did not extend normally and the Vmd and

Fig. 1. Hs6st1 and Hs6st2 regulate cranial axon guidance, but not FBM neuron migration. (A) 11.5 dpc hindbrain explants were treated with inhibitors or implanted with beads containing growth factors. (B) Hindbrain explants (n=4) were treated with vehicle or heparitinase and immunostained for ISL1. Asterisks indicate the midline. (C) Schematic representation of FBM neuron migration and ISH of 12.5 dpc hindbrains to reveal Hs6st1 and Hs6st2 expression relative to Isl1-positive migrating FBM neurons (VIIin) and the paired facial motor nuclei (VIIin). (D) Isl1 ISH of Hs6st1−/− (n=3), Hs6st2−/− (n=10), Hs6st1−/−;Hs6st2−/− (minor defects in 2/5 mutants examined) and wild-type (n=6) hindbrains; the arrow indicates a minor defect in FBM neuron migration. (E) Lateral view of 11.5 dpc Hs6st1−/−, Hs6st2−/− and Hs6st1−/−;Hs6st2−/− mutant (n=3 each) and wild-type (n=5) heads after immunolabelling for TUJ1. Green arrows indicate delayed extension of the mandibular (Vmd) and maxillary (Vmx) nerves from the trigeminal ganglion (Vg), red arrows indicate abnormal chorda tympani (VIIct) and facial branchiomotor nerve (VIIbm) branches from the facial ganglion (VII) and open triangles the lack of ophthalmic (Vop) and greater superficial petrosal (VIIgspn) nerves. Scale bars: 200 μm in B,C,E; 100 μm in D.
Vop nerves were absent in three out of three Hs6st1−/− Hs6st2−/− double mutants (Fig. 1E).

These results demonstrate that 6-O-sulfotransferases are dispensable for FBM neuron migration, but essential for cranial nerve axon guidance.

6-O-sulfotransferases modulate trigeminal axon extension in vitro

The trigeminal axon defects of Hs6st1−/− Hs6st2−/− mutants resembled those of Slit mutants (Ma and Tessier-Lavigne, 2007). To investigate whether these 6-O-sulfotransferases are required for SLIT proteins to modulate trigeminal axon extension, we explanted trigeminal ganglia to observe neurite extension in the absence or presence of SLIT proteins (Fig. S1). In culture media lacking exogenous SLITs, 6-O-sulfotransferase deficiency increased the number of neurites (Fig. S1A,B) and the maximal distance of neurite extension from the explant (Fig. S1A,C). Moreover, compound mutant explants extended unusually long and thick neurites with a non-linear growth pattern that were rarely present in wild-type and single mutant explants (Fig. S1A). These findings corroborate that 6-O-sulfotransferases modulate trigeminal axon growth. However, 6-O-sulfotransferases were inhibitory in this assay, even though they are required for axon path-finding in vivo (Fig. 1E). This observation raises the possibility that explant cultures lack factors that alter 6-O-sulfotransferase-dependent processes from inhibitory to growth-promoting. Recombinant SLIT2, but not SLIT1, reduced neurite extension from wild-type explants (Fig. S1D,E). SLIT2 also reduced neurite extension from mutant explants to a similar extent as from wild-type explants (Fig. S1F,G). Moreover, SLIT2 did not decrease the formation of aberrant long and thick neurites in compound mutant explants (Fig. S1F). Thus, SLIT2 and 6-O-sulfotransferases regulate neurite extension independently of each other, and Hs6ST1 and Hs6ST2 promote cranial axon guidance in pathways that remain to be identified.

Hs2st is essential for FBM neuron migration, but dispensable for cranial axon guidance

To determine the expression pattern of HS2ST during hindbrain development, we used mice carrying an Hs2stLacZ knock-in allele that recapitulates endogenous Hs2st expression when visualised as β-galactosidase-mediated X-gal staining (Bullock et al., 1998). Hs2stLacZ/− hindbrains at 10.5 dpc showed prominent staining in r4, close to the domain in which FBM neurons differentiate (Fig. 2A). The Hs2st expression domain was adjacent to, but did not overlap with, the area that contains FBM neurons (Fig. 2B). By 12.5 dpc, Hs2st expression seemed to be restricted to the midline region (Fig. 2C). These expression patterns raise the possibility that HS2ST

Fig. 2. Hs2st is expressed near FBM neurons and is essential for their appropriate migration. (A) Whole-mount X-gal staining of 10.5 dpc Hs2stLacZ/− hindbrains (n=5) shows Hs2st expression in r4 (arrow) adjacent to the motor column, visualised by Isl1 ISH (arrowhead). (B) Transverse 10.5 dpc Hs2stLacZ/− sections through r4 level were X-gal stained and then immunostained for ISL1 to demonstrate Hs2st expression adjacent to FBM neurons (VII, the X-gal stain was pseudocoloured red in the right-hand panel). The arrow indicates Hs2st expression. (C) Whole-mount X-gal staining of 12.5 dpc Hs2stLacZ/− hindbrains (n=3) shows Hs2st expression in the midline area (red wavy arrow). (D) Isl1 whole-mount ISH of Hs2stLacZ/− and wild-type hindbrains (n=6 each); brackets indicate the width of the FBM neuron stream. (E) Lateral views of 11.5 dpc Hs2stLacZ/− heads and controls, immunolabelled for TUJ1 (n=3 each). The Vop, Vop, and Vop nerves extend from the trigeminal ganglion (V9), and the VII, VII, and VII, nerves from the facial ganglion (VIIa) in both genotypes. Scale bars: 200 μm in A, C,E, 400 μm in D; 50 μm in B.
modifies proteoglycans that regulate FBM neuron development in trans. To determine whether HS2ST is required for FBM neuron migration, we examined Hs2st-null (Hs2st<sup>Lacz/Lacz</sup>) hindbrains by IISL ISH. We observed splitting of the migratory stream in all mutants examined (Fig. 2D). By contrast, whole-mount TUJ1 immunolabelling showed normal axon extension of all facial and trigeminal nerve branches in mutants (Fig. 2E). These results suggest that 2-O-sulfotransferase is important for FBM neuron migration, but not cranial axon guidance.

**Hs2st is dispensable for VEGF-mediated FBM neuron migration**

HSPGs interact with VEGF and its receptor NRP1 (Sarrazin et al., 2011). We therefore asked if loss of 2-O-sulfated HSPGs caused an FBM neuron defect similar to that caused by loss of VEGF signalling through NRP1 (Schwarz et al., 2004). Comparable to Nrp1-null mice and Vegfa<sup>120/120</sup> mice lacking NRP1-binding VEGF (Schwarz et al., 2004), migrating FBM neurons split into two main streams in Hs2st-null hindbrains (Fig. 3A). To examine whether HS2ST-modified HSPGs were required for VEGF-induced FBM neuron migration, we implanted VEGF<sub>165</sub>-coated heparin beads into Hs2st-null and littermate control hindbrains. As previously shown (Schwarz et al., 2004), FBM neurons migrated towards the beads in controls (Fig. 3B). Unexpectedly, these neurons also migrated towards the beads in Hs2st-null hindbrains (Fig. 3B). Quantification confirmed that VEGF<sub>165</sub> beads promoted FBM neuron migration in both genotypes (Fig. 3B). Despite the similar in vivo phenotypes of Hs2st-null and Vegfa<sup>120/120</sup> mice, HS2ST-modified HSPGs are therefore not required for VEGF-induced FBM neuron migration.

**FGF can promote FBM neuron migration in an HS2ST-dependent fashion**

HSPGs act as FGF co-receptors and regulate the distribution and degradation of FGFs (Hacker et al., 2005). Microarray analysis showed that FGF3-5, 8, 9, 11-15 and 17-23 as well as the four FGF receptors (Fgfr1-4) were expressed in 11.5 dpc hindbrains (Fig. 4A). Reverse transcription PCR (RT-PCR) confirmed Fgfr1-4 expression in 11.5 dpc hindbrains (Fig. 4B). ISH showed that Fgfr1-4 were expressed widely at 10.5 dpc, the onset of FBM neuron migration, whereas 12.5 dpc, Fgfr1-3 were expressed near the midline and Fgfr4 was expressed by FBM neurons when they reached r6 (Fig. 4C). To examine whether FBM neurons can in principle respond to FGF ligands, we performed hindbrain explants with FGF-coated beads. For these experiments, we used FGF2, because it is known to bind all four FGF receptors and requires HSPGs for appropriate signalling (Matsuo and Kimura-Yoshida, 2013). FBM neurons in wild-type hindbrains were strongly attracted by FGF2 beads, but attraction was abolished in Hs2st-null hindbrains (Fig. 4D). Quantification confirmed significantly impaired FGF2-induced FBM neuron migration in the mutants (Fig. 4D). These observations suggest that HSPGs can regulate FGF-induced FBM neuron migration in vivo.

As Hs2st is prominently expressed in r4 at 10.5 dpc, we considered the possibility that HSPGs modulate FGF-induced signalling pathways in this rhombomere to regulate FBM neuron migration. We observed that Erm (also known as En5), a member of the ETS transcription factor family that is positively regulated by FGF (Raible and Brand, 2001; Firnberg and Neubuser, 2002), was expressed in r6-r8 along the midline at 10.5 dpc (Fig. 4E). Notably, 10.5 dpc Hs2st-null hindbrains upregulated Erm expression in the midline region of r2 and r4, the rhombomeres that give rise to the trigeminal branchiomotor and FBM neurons, respectively (Fig. 4E). This finding agrees with prior observations that HSPGs can spatially restrict the diffusion or degradation of FGF ligands in the zebrafish embryo to modulate FGF signalling (Venero Galanterniki et al., 2015). In contrast to Erm, the master regulator of r4, Hoxb1, was expressed normally in 10.5 dpc Hs2st-null hindbrains (Fig. 4F).

**Fig. 3. VEGF does not require HS2ST to control FBM neuron migration.** (A) Transverse sections through 12.5 dpc r6, immunolabelled for ISL1, TUJ1 and the vascular marker IB4; arrows indicate similar stream splitting of FBM neurons in Hs2st<sup>Lacz/Lacz</sup>, Nrp1<sup>−/−</sup> and Vegfa<sup>120/120</sup> mice (n=3 each). (B) Hindbrain explants containing VEGF<sub>165</sub> beads (position indicated with red circles) were immunostained for ISL1; red arrows indicate the leading edge of FBM neurons migrating towards the beads. The distance migrated on the implanted relative to the control side of each hindbrain is shown as mean±s.e.m. Hs2st<sup>−/−</sup>, n=15; Hs2st<sup>Lacz/Lacz</sup>, n=7. *P<0.05, **P<0.01, paired t-test. Scale bars: 200 μm.
This observation suggests that r4 is specified normally in Hs2st-null mutants and agrees with Erm upregulation resulting from HS2ST-dependent local alterations in FGF signalling. Together, our findings are consistent with a role for HS2ST in FGF-mediated neuronal guidance and/or hindbrain patterning. Future studies might investigate whether HS2ST also regulates other molecules that control FBM migration non-cell autonomously, such as cadherins and PCP pathway components (Vivancos et al., 2009; Qu et al., 2010; Stockinger et al., 2011; Zakaria et al., 2014).

**Conclusion**

Hs6st1 and Hs6st2 synergise to regulate cranial axon guidance whilst Hs2st promotes FBM neuron migration. Thus, HS6ST1/2 and HS2ST have distinct, but complementary, roles in cranial nerve development.

**MATERIALS AND METHODS**

**Animals**

Animal procedures were performed in accordance with institutional guidelines under the authority of a UK Home Office project licence. Mice were paired in the evening, and the morning of vaginal plug formation counted as 0.5 dpc. We used Hs2stLacz/+(Bullock et al., 1998), Hs6st1+/− (Leighton et al., 2001; Mitchell et al., 2001), Hs6st2+/− (Qu et al., 2011) and Vegfa120/+(Carmeliet et al., 1999) mice on a C57/Bl6 background and Nrp1+/− (Kitsukawa et al., 1997) mice on a CD1 background.

**Microarray and RT-PCR analysis**

Microarray analysis was performed with the GeneChip Mouse 1.0 ST Array (Affimetrix) on 11.5 dpc Spi1+/− and Spi1+/+ hindbrains (n=3 each); expression was measured as optical density for each probe set, values were normalised by robust multi-array analysis and transformed to a log2 scale.


