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Distinct expression patterns of *syndecans* in the embryonic zebrafish brain

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ABSTRACT

Axon pathfinding in the neuroepithelium of embryonic brain is dependent on a variety of short and long range guidance cues. Heparan sulfate proteoglycans such as syndecans act as modulators of these cues and their importance in neural development is highlighted by their phylogenetic conservation. In Drosophilia, a single syndecan is present on the surface of axon growth cones and is required for chemorepulsive signalling during midline crossing. Understanding the role of syndecans in the vertebrate nervous system is challenging given that there are four homologous genes, syndecans 1–4. We show here that syndecans 2–4 are expressed in the zebrafish embryonic brain during the major period of axon growth. These genes show differing expression patterns in the brain which provides putative insights into their functional specificity.

Syndecan proteoglycans are single-pass transmembrane proteins with heparan sulphate and chondroitin sulphate chains covalently attached to their extracellular domain. They have been linked to numerous biological processes including neural patterning and axon guidance (Bass et al., 2009). In mammals there are four syndecan *(sdc)* genes (1–4), while there is only a single orthologue present in invertebrates. Interestingly, in zebrafish only homologues of *sdc 2–4* have been identified (Fig. 1 and Supplementary Table 1). The homologue for *sdc1* appears to have been lost from the zebrafish genome since the separation of the mammalian and fish lineages (Chakravarti and Adams, 2006). In zebrafish, syndecans (Sdc) *2–4* all play a role in epiboly (Lambaerts et al., 2012) while Sdc2 and 4 (Oh and Couchman, 2004), which show a high degree of structural similarity (Oh and Couchman, 2004), are involved in a number of distinct developmental processes (Arrington and Yost, 2009; Chen et al., 2004; Matthews et al., 2008). For instance, Sdc4, participates in neural crest migration (Matthews et al., 2008) while Sdc2 modulates both angiogenic sprouting (Chen et al., 2004) and migration of organ primordia (Arrington and Yost, 2009).

Heparan sulphate proteoglycans such as syndecans, glypicans and perlecans, can modulate development of the central nervous system (CNS) by binding to and altering the activity (either directly or indirectly) of axon guidance molecules (e.g. Slits (Liang et al., 1999)) as well as morphogens such as Shh, FGFs and Wnts (Gunhaga et al., 2003; Lee et al., 2000; Viti et al., 2003). Knock down of the heparan sulfation enzymes *Ndst1* and *Ext1* in mice causes abnormalities in various brain structures, including forebrain commissures (Grobe et al., 2005; Inatani et al., 2003). At present the role of the individual syndecan genes in CNS development remains unknown. Despite the advantages of using zebrafish as a model system to study development of the nervous system, the neural expression patterns of the syndecan genes remain unknown. We used wholemount fluorescence *in situ* hybridization combined with immunostaining for axon tracts to characterise the spatial expression patterns of the syndecans in the embryonic zebrafish brain. We found that *sdc2, sdc3* and *sdc4* are expressed in distinct and overlapping regions of the brain consistent with redundant and non-redundant roles in brain development.

1. Results and discussion

1.1. Expression of syndecan 2

Given the role of syndecan (*sdc*) in slit/robo mediated axon guidance in Drosophila (Johnson et al., 2004) and our recent reports of the involvement of *robos* and *slits* in longitudinal and commissural axon growth in the zebrafish forebrain (Devine and Key, 2008; Hofmeister et al., 2012), we undertook to examine the expression of *sdcs* in the embryonic zebrafish brain. Initially we used brightfield microscopy to visualise *sdc2 in situ* hybridization in whole mounts of 28 hours post-fertilization (hpf) embryonic brains stained by NBT/BCIP immunohistochemistry (Supplementary Fig. 1). However, because of weak and diffuse reaction products in the brain it was difficult to accurately discern its spatial expression pattern. This weak staining was not due to inefficient binding of the RNA probe as a strong signal was observed in the hypocord (unfilled arrow, Supplementary Fig. 1D) and axial vasculature as previously observed (Chen et al., 2004). In order to better visualise the distribution of *sdc2* in relation to major brain landmarks we next examined expression using fluorescent *in situ* hybridisation in conjunction with antibody staining for the

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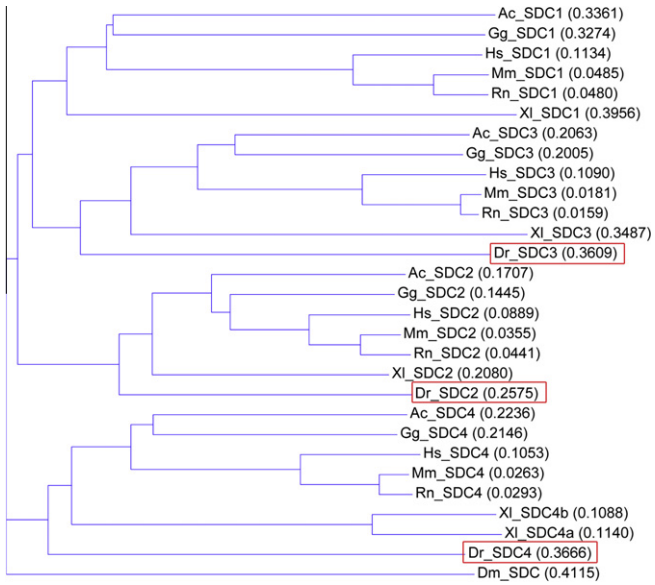

Fig. 1. Phylogenetic tree showing alignment of syndecan proteins. Protein sequences were aligned from lizard (*Anolis carolinensis*, Ac), zebrafish (*Danio rerio*, Dr), fruit fly (*Drosophila melanogaster*, Dm), chicken (*Gallus gallus*, Gg), human (*Homo sapiens*, Hs), mouse (*Mus musculus*, Mm), rat (*Rattus norvegicus*, Rn), frog (*Xenopus laevis*, Xl). Accession numbers are provided in Supplementary Table 1.

pan-axonal marker acetylated α-tubulin (Fig. 2), a sense control probe was used to control for non-specific staining (Supplementary Fig 5A–C). By 24–28 hour post-fertilization (hpf), anti-acetylated α-tubulin stains a highly stereotypical scaffold of axon tracts (Hjorth and Key, 2002). These tracts arise from a set of nuclei that seed the major subdivisions of the brain. When serial optical sections of the lateral view of the brain were captured by laser confocal microscopy and compiled it was possible to clearly observe the spatial pattern of expression of *sdc2* in relation to the major axon tracts (Fig. 2A). *Sdc2* was clearly expressed in two distinct regions of the brain: a rostrodorsal diencephalic patch of neuroepithelium (asterisk, Fig. 2A; unfilled arrow in Fig. 1B) and in the isthmus, a major signalling zone lying at the midbrain–hindbrain boundary (dashed yellow outline, Fig. 2A) (Lun and Brand, 1998). At the isthmus, staining is strongest adjacent to the midbrain ventricle (unfilled arrowhead Fig. 2B and H). By examining individual optical scans (Supplementary Fig. 2) it was possible to follow the isthmus expression pattern from the lateral surface of the brain (Fig. 2D–F; unfilled arrowhead Supplementary Fig. 2B) towards the midline (Fig. 2G–I; unfilled arrowhead Supplementary Fig. 2N).

Weak expression could also be traced in the neuroepithelium joining the dorsal diencephalic patch (asterisk, Fig. 2A) with the tegmentum (small filled arrowheads, Fig. 2B; dotted line, Supplementary Fig. 2H, K, and N). This region of the brain contains the caudal portion of the tract of the post-optic commissure which connects the ventral diencephalon and the tegmentum and the rostral portion of the ventrolateral longitudinal axon tract (arrows, Fig. 2A). *sdc2* was not expressed in either the telencephalon or the cerebellum at this age (T, C in Fig. 2A).

1.2. Expression of syndecan 3

Next we examined the expression pattern of *sdc3* in the 28 hpf embryonic zebrafish brain (Fig. 3, and Supplementary Fig. 3, for sense control see Supplementary Fig 5D–F). *sdc3* was more widely expressed in the neuroepithelium in comparison to *sdc2*. In particular, *sdc3* was present throughout the ventrodorsal diencephalon (red dotted outline, Fig. 3B) and ventral telencephalon (green dotted outline, Fig. 3B). It was noticeably absent from both the rostral dorsal telencephalon (also referred to as pallium) (asterisk, Fig. 3A) and a dorsoventral band of neuroepithelium within the middle of the diencephalon (filled arrowhead, Fig. 3A). The expression of *sdc3* was closely associated with some axon tracts such as the anterior tract of the post-optic commissure (POC, Fig. 3A and D), the tract of the posterior commissure (TPC, Fig. 3A–C) and the supra-optic tract (SOT) (Supplementary Fig. 3G–I). While *sdc3* is aligned with axon tracts in rat (Kinnunen et al., 1998), we found that *sdc3* was not ubiquitously expressed in the neuroepithelium underlying all axon tracts in zebrafish embryonic brain. Interestingly, *sdc3* was absent from the trajectory of the caudal portion of the TPOC (filled arrow, Fig. 3A).

Given that various growth factors, morphogens and axon guidance molecules known to interact with syndecans also play a role in the formation of the anterior and post-optic commissures (Rhiner and Hengartner, 2006), we next examined in more detail the expression of syndecans in the commissural plate that forms the rostral end of the brain (Johnston, 1909; Muller and O'Rahilly, 1986; Smith, 1903). Compiled coronal optical slices of the commissural plate reveals the crossing of axons within the anterior commissure (AC, Fig. 3D) and the post-optic commissure (POC, Fig. 3D). *sdc3* was more strongly expressed in the ventral half of the plate, beginning from the optic recess (OR; Fig. 3D–F). *sdc3* was also present, albeit weakly in the neuroepithelium associated with the anterior commissure. This expression pattern is consistent with *sdc3* playing a role in the development of these important axon pathways that link the two sides of the rostral brain.

Next we examined *sdc3* expression earlier in development when the very first axons are initially pathfinding in the neuroepithelium. At 18 hpf, pioneer axons are forming the tract of the post-optic commissure in the rostroventral diencephalon (unfilled arrowheads, Fig. 3G and J) and the medial longitudinal fascicle within the midbrain tegmentum (unfilled arrow, Fig. 3G). At low magnification, *sdc3* is expressed in the neuroepithelium surrounding these early forming axon tracts (Fig. 3G–L). When viewed at higher magnification, individual neuronal perikarya in the ventral diencephalon can be seen extending pioneer axons along the presumptive tract of the post-optic commissure in a region expressing *sdc3* (Fig. 3M–O). Interestingly, at this age the epiphysis in the dorsal diencephalon is strongly expressing *sdc3* (filled arrowhead, Fig. 3H). *sdc3* is also expressed in the ventral or sub-pallial telencephalon (asterisks, Fig. 3G and J) in the region where neurons will emerge and give rise to the anterior commissure.

Taken together, these results indicate that *sdc3* is associated with the pathways of a subset of axon tracts in the brain. *sdc3* is expressed during critical stages of neural development including the period before axon tract formation, during pioneer axon outgrowth and while axon tracts are undergoing extensive expansion.

1.3. Expression of syndecan 4

At 28hpf, while *sdc4* expression (Fig. 4 and Supplementary Fig. 5G–I for sense control) shares some similarity to that of *sdc2*, it is clearly distinct from *sdc3*. As for *sdc2*, *sdc4* is expressed by the isthmus (unfilled arrow, Fig. 4A). *sdc4* was expressed by a patch of neuroepithelium in the rostral diencephalon (asterisk, Fig. 4A, D, and J) that was linked via a longitudinal stripe of cells (filled arrowheads, Fig. 4D) to a band of cells in the dorsal diencephalon (unfilled arrow, Fig. 4D). These dorsal diencephalic cells are connected by another stripe of cells (filled arrowheads, Fig. 4B) to the isthmus. Both the tectum (Tt, Fig. 4A) and the telencephalon (T, Fig. 4B), did not express *sdc4*. Weak diffuse expression of *sdc4* was observed in the ventral diencephalon (vD, Fig. 3B) and tegmentum (Tm, Fig. 4B). In contrast to *sdc3*, *sdc4* did not appear to