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Dual function of polysialic acid during zebrafish central nervous system development

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SUMMARY

Polysialic acid (PSA), a carbohydrate epitope attached to the neural cell adhesion molecule, serves as a modulator of axonal interactions during vertebrate nervous system development. We have used PSA-specific antibodies and whole-mount immunocytochemistry to describe the spatiotemporal expression pattern of PSA during zebrafish central nervous system development. PSA is transiently expressed on all cell bodies and, except for the posterior commissure, it is not found on axons. Floorplate cells in the spinal cord and hindbrain strongly express PSA throughout development. Enzymatic removal of PSA leads to a defasciculated growth pattern of the posterior commissure and also affects distinct subsets of commissural axons in the hindbrain, which fail to cross the midline. Whereas the disordered growth pattern of hindbrain commissures produced by PSA-removal could be mimicked by injections of soluble PSA, the growth of axons in the posterior commissure was unaffected by such treatment. These results suggest that there are distinct mechanisms for PSA action during axon growth and pathfinding in the developing zebrafish CNS.

Key words: Carbohydrate, NCAM, Axon guidance, Commissural axon, Floorplate, Midline, Zebrafish, *Danio rerio*

INTRODUCTION

Polysialic acid (PSA) is a long linear homopolymer of negatively-charged sialic acid that in vertebrates is attached to the neural cell adhesion molecule, NCAM. In the developing vertebrate nervous system, the abundant and highly regulated expression of PSA is correlated with periods during which cells migrate and axons seek and contact their targets (Kiss and Rougon, 1997; Rutishauser, 1998). In the adult nervous system, PSA expression is more restricted and primarily associated with regions capable of morphological and/or functional plasticity (Seki and Arai, 1991). Studies of the role of PSA have been facilitated by an endo Neuraminidase (endo N) derived from bacteriophage. Endo N specifically degrades PSA but does not affect other sialic acid-containing structures (Hallenbeck et al., 1987). Using endo N, it has been shown that PSA-NCAM is involved in a variety of morphogenetic processes in the developing nervous system, including migration of neural precursors (Ono et al., 1994; Hu et al., 1996; Wang et al., 1994), axon outgrowth (Doherty et al., 1990; Zhang et al., 1992) and the branching of neurites in response to guidance or targeting signals in their environment (Tang et al., 1992; Yin et al., 1995; Daston et al., 1996).

PSA has the ability to weaken not only NCAM-mediated adhesion but also a variety of cell-cell interactions mediated by other molecules (Rutishauser and Landmesser, 1996). Its global effect on cell interactions includes an overall steric influence of PSA on membrane-membrane apposition (Yang et al., 1992). Additional mechanisms for PSA action have been proposed. For example, the ability of endo N to block induction of long-term potentiation and long-term depression in hippocampal slice cultures (Muller et al., 1996) has been attributed to an effect of PSA on the activity of brain-derived neurotrophic factor (Muller et al., 2000). There also is evidence from in vitro experiments that the ability of NCAM to bind to a matrix containing heparan sulphate proteoglycans is augmented by PSA (Storms and Rutishauser, 1998). In both of these cases, it is suggested that PSA has a specific binding affinity that can be competitively inhibited by PSA that is not attached to NCAM (that is, soluble and isolated PSA chains as found in colominic acid). This ability of soluble PSA to mimic the action of endo N, which does not occur with the steric effect of PSA on cell-cell interactions (Storms and Rutishauser, 1998; Muller et al., 2000), is therefore characteristic of a positive binding mode of PSA action.

The polysialylation of NCAM occurs in all vertebrates but appears to be absent in invertebrates (Rutishauser, 1998). However, nearly all studies of PSA function have been carried out in higher vertebrates, such as mammals and birds (Kiss and Rougon, 1997). We have chosen the zebrafish embryo for investigation of the function of PSA during development of the nervous system of lower vertebrates. In the past decade,

embryonic zebrafish have become a popular model for studying the cellular and molecular bases of nervous system development (Eisen, 1996) and axonal pathfinding (Bernhardt, 1999). The anatomy of the embryonic nervous system has been described in detail (Chitnis and Kuwada, 1990; Wilson et al., 1990; Mendelson, 1986; Metcalfe et al., 1986) and the accessibility of the embryos allows for functional in vivo experiments (Weiland et al., 1997; Lauderdale et al., 1997; Bernhardt and Schachner, 2000; Ott et al., 2001).

In the present study, we have used PSA-specific antibodies and whole-mount immunocytochemistry to describe the spatiotemporal expression pattern of PSA during zebrafish CNS development. Injections of endo N into the developing CNS caused a disordered growth pattern of commissural axons in the hindbrain and the posterior commissure (pc). Whereas the disordered growth pattern of hindbrain commissures produced by endo N could be induced by injections of soluble PSA, the growth of axons in the pc was unaffected by such treatment. The results suggest that there are distinct mechanisms for PSA action during axon growth and pathfinding in the developing zebrafish CNS.

MATERIALS AND METHODS

Animals

Zebrafish embryos of the golden strain were obtained from our laboratory colony and maintained at 28.5°C. Embryos were staged by hours postfertilisation (hpf). Dechorionated embryos were anaesthetised in 0.03% aminobenzoic acid ethyl ester (MS222; Sigma) before injection and fixation.

Purification of brain membranes

A membrane-enriched fraction of normal adult goldfish brain homogenate was obtained as described (Vielmetter et al., 1991). In brief, the brains were homogenised in homogenisation buffer (pH 7.4, 10 mM Tris-HCl, 1.5 mM $CaCl_2$, 15 μg/ml 2,3-dehydro-2-desoxy-N-acetylneuraminic acid) containing the protease inhibitors spermidin (1 mM), aprotinin (25 μg/ml), leupeptin (25 μg/ml) and pepstatin (5 μg/ml). Cell surface membranes were enriched in the interband of a sucrose step gradient (upper phase 20%, lower phase 50% sucrose) by centrifugation (6×10^4 ***g***, 10 minutes, 4°C). The membrane fragments were solubilised in buffer with the detergent octylglucoside (OG lysis buffer: 100 mM octylglucosid, 20 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml phenylmethylsulfonyl fluoride, pH 7.4) by sonication. The supernatant obtained after centrifugation (10^5 ***g***, 1 hour, 4°C) was called OG-brain extract. OG-brain extract from zebrafish was obtained in the same way as described above.

Purification of PSA glycoproteins

Immunoaffinity absorption of the glycoproteins was performed using a column of CNBr-Sepharose (Pharmacia) to which the monoclonal anti PSA antibody 735 had been covalently coupled (kindly provided by Rita Gerardy-Schahn). Goldfish OG-brain extract was passed over the affinity column (12 hours at 4°C under rotation). The affinity column containing the bound antigen was washed with OG lysis buffer followed by OG lysis buffer containing 0.5M NaCl, and then with pre-elution buffer (20 mM Tris-HCl, 150 mM NaCl, 100 mM octylglucosid, pH 8). The antigen was eluted with elution buffer (100 mM triethylamine, 100 mM octylglucosid, pH 11.5) into 2 M Tris-HCl, pH 6 (50 μl per 250 μl eluate) to neutralise the eluate.

The eluted antigen was subjected to SDS 8% PAGE under nonreducing conditions. For western blot analysis, the antigen was transferred to a nitrocellulose membrane (Hybond-C extra, Amersham), blocked with 3% fat-free milk powder in phosphate-buffered saline (PBS) and 0.05% Tween (1 hour, 37°C), incubated (overnight, 4°C) with monoclonal antibodies against PSA (mAb 735) or NCAM (mAb D3 (Schlosshauer, 1989)). After three washes (5 minutes each) with PBS containing 0.05% Tween, antibody binding was detected by horseradish peroxidase-coupled goat anti-mouse antibodies (Dianova, diluted 1:10000 in 3% milk powder, 0.05% Tween in PBS), and developed in staining solution (Super Signal, Pierce). For Silver stain analysis, the eluted PSA glycoproteins were digested with endo N (1 μg/ml eluate, 2 hours at 37°C).

Production of polyclonal antibodies against PSA glycoproteins

Polyclonal antibodies were produced by injecting rabbits subcutanously with approximately 3 μg of purified PSA glycoprotein three times at intervals of three weeks. The antigen was emulsified in adjuvant (MPL+TDM+CWS Adjuvant system, Sigma) according to the manufacturer's protocol. Serum was collected on the eighth day after the third injection and IgG antibodies were purified with a protein A sepharose column (Pharmacia).

Antibody staining

Zebrafish embryos were processed for whole-mount immunohistochemistry as previously described (Weiland et al., 1997). Briefly, embryos were fixed for 4 hours at 4°C in 4% paraformaldehyde in fixation buffer (Westerfield, 1994) and permeabilised by exposure to acetone (–20°C for 2-5 minutes). After incubation in blocking buffer (PBS with 1% bovine serum albumin (BSA), 1% DMSO, 2% goat serum, 2% donkey serum) for 1 hour at 37°C, they were exposed to primary antibodies overnight at 4°C.

In this study, three monoclonal antibodies against PSA were used: mAb 735 (an IgG) (Frosch et al, 1985), mAb 5A5 (an IgM) (Dodd et al, 1988) and mAb 12E3 (an IgM) (Seki and Arai, 1991). The specificity of the antibodies was tested on whole-mount staining with Endo N-injected embryos. In contrast to control embryos, which show a distinct staining pattern, there was no staining in embryos treated with endo N, indicating that the antibodies are specific for PSA and do not crossreact with other epitopes in fish material.

To visualise zebrafish axons, a monoclonal antibody against acetylated α-tubulin (25 μg/ml, clone 6-11B-1, Sigma) and a polyclonal serum against zebrafish Tag-1 (Lang et al., 2001) were applied. Embryos were rinsed in wash buffer (PBS with 1% BSA, 1% DMSO) and incubated with either Alexa-488-coupled goat anti-rabbit (2 μg/ml; Molecular Probes), cyanin-3-coupled donkey anti-mouse IgG (H+L) (2 μg/ml; Dianova), or cyanin-3-coupled goat anti-mouse IgM, μ-chain specific (2 μg/ml, Dianova) for 1 hour at 37°C. Yolk sacs were removed and embryos were embedded between two coverslips in Mowiol containing n-propylgallate as an antifading agent.

DiI labeling

Embryos were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight and embedded in 1% low melting point agarose (Sigma) in PBS. Excess agarose covering the hindbrain was removed. The tip of a pulled glass micropipette was dipped into a 1% DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, Molecular Probes) solution in ethanol and inserted into the dorsolateral aspect of the hindbrain, rostral to the otocyst. Embryos were stored in a wet chamber overnight at 4°C to allow diffusion of DiI along commissural axons. For analysis, embryos were re-embedded in 0.3% agarose (Gibco) in PBS and analysed with a confocal microscope.

Enzyme injections

Dechorionated embryos were oriented in a specialised sylgard matrix (Ott et al., 2001) on a heated stage at 28.5°C, allowing for the efficient injection of 6×30 animals. Pulled 5 μl glass micro pipettes were broken under optic control to short tips of 8 μm diameter and filled with solution using negative pressure. The pipette was manually

inserted into the ventricle of the embryos and approximately 10 nl of solution was pressure injected using a microinjector (Eppendorf Transjector 5246). We injected either endo N (60 μg/ml, kindly provided by Rita Gerardy-Schahn), soluble PSA (colominic acid, 100 mg/ml in PBS, ICN Biomedicals), PSA-Trimer (N-Acetylneuraminic acid, Trimer, 100 mg/ml in PBS, Calbiochem) or control buffer (PBS). Single injections were performed at 20 hpf; embryos were fixed for immunocytochemistry at 32 hpf and for DiI labeling at 36 hpf.

Analysis of stained embryos

Stained embryos were analysed using a confocal microscope (LSM 510, Zeiss) equipped with a high aperture lens (C-Apochromat 40×/1.2W, Zeiss) and the appropriate lasers. Serial optical sections were flattened into projections. All images were further processed with Adobe Photoshop 5.02 software.

RESULTS

Distribution of PSA during zebrafish development

To determine the spatiotemporal expression pattern of PSA during zebrafish development, embryos aged between 16 hpf and 5 days were subjected to whole-mount immunocytochemistry. The three monoclonal anti-PSA antibodies tested all produced a similar staining pattern during zebrafish development. PSA is first detectable at 17 hpf (Fig. 1A). Counterstain with an antibody against the cell recognition molecule Tag1 shows that at this time the first neurones and axons have differentiated (Fig. 1A). PSA staining at this stage is weak, uniform throughout the CNS and, as in later stages, associated with membranes of almost all cell bodies (Fig. 1B). With further development, the overall staining intensity of PSA increases and reaches its peak between 27-40 hpf. Embryos at 27 hpf, double-labelled for PSA (Fig. 1C) and acetylated tubulin as an axonal marker (Fig. 1D), reveal that PSA is highly expressed on branchiomotor nuclei V and VII in the hindbrain and on their axons leaving the CNS. But, in contrast to higher vertebrates, most other axon tracts in the developing CNS are not positive for PSA. A notable exception are axons in the posterior commissure (pc), which express high amounts of PSA from 27 hpf onwards (Fig. 1E,F). Floorplate (fp) cells in the spinal cord (Fig. 1H) and hindbrain (Fig. 1G) are also strongly positive for PSA. Expression on floorplate cells begins

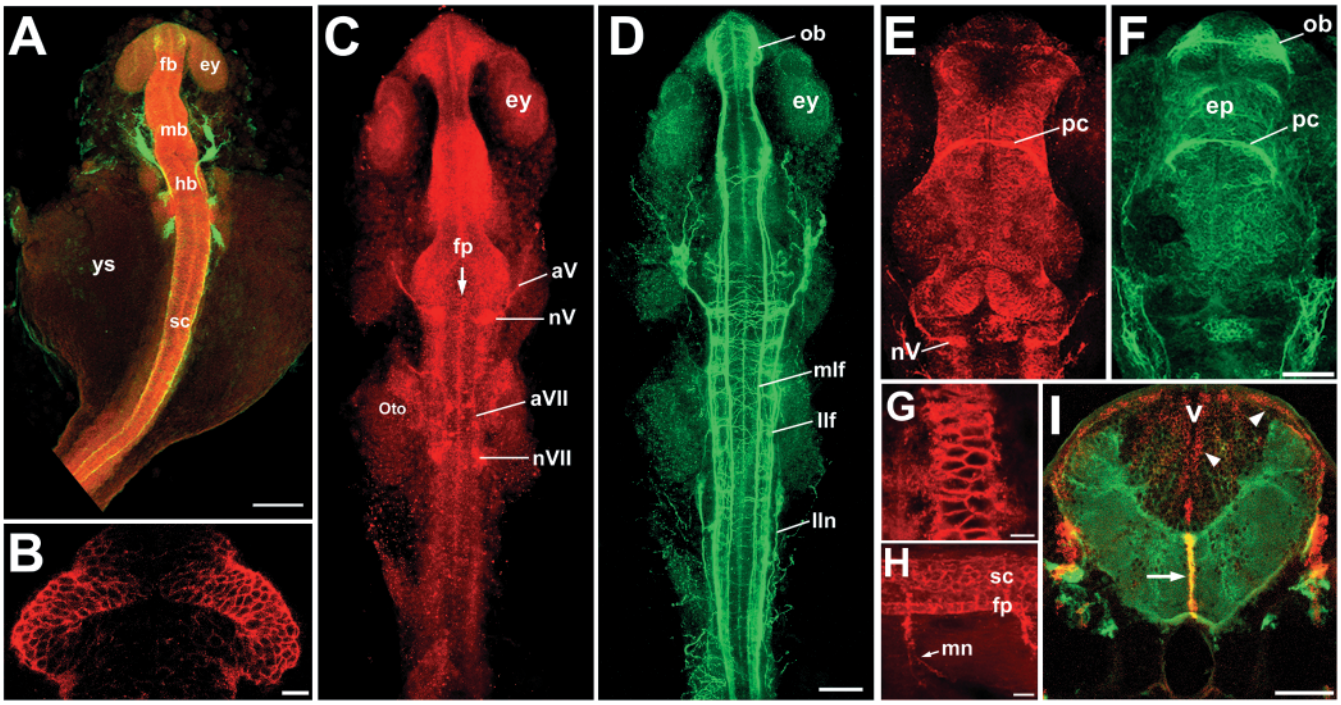

Fig. 1. Expression pattern of PSA during zebrafish development. (A) Embryo at 17 hpf, labelled with antibodies against PSA and Tag-1. PSA labeling (red) is uniform throughout the CNS and not found on the first differentiated Tag-1-positive axons (green). (B) Single optical section through the CNS of a 27 hpf embryo demonstrating the association of PSA with membranes of almost all cell bodies. (C,D) Embryo at 27 hpf double labelled with antibodies against PSA and α-tubulin as an axonal marker. (C) PSA is expressed on cell bodies in the CNS, on floorplate cells (fp) along the midline, the nuclei of cranial nerves V and VII (nV, nVII) and on their axons (aV, aVII) leaving the CNS. (D) Counterstain with α-tubulin demonstrates that most axon tracts, including the medial longitudinal fascicle (mlf), the lateral longitudinal fascicle (llf) and the lateral line nerve (lln) are PSA negative. (E,F) Embryo at 30 hpf double labelled with antibodies against Tag-1 and PSA. (E) Axons in the posterior commissure (pc) express high levels of PSA, whereas the cells in the environment show lower levels of PSA. (F) The Tag-1 staining demonstrates that axons in the olfactory bulb (ob) and the epiphysis (ep) are PSA-negative. (G) Hindbrain of an embryo at 32 hpf with strong PSA expression on floorplate cells along the midline. (H) Lateral view of the tail of an embryo at 36 hpf showing PSA expression on cell bodies in spinal cord (sc), the floorplate (fp) and on secondary motor axons (mn) (Ott et al., 2001) leaving the CNS. (I) Transverse cryosection through the hindbrain of a fish larva at 13 days, double labelled for PSA (red) and Tag-1 (green). PSA staining is restricted to cells close to the ventricle and the pial surface (arrowheads), and a strong signal along the midline (arrow). ep, epiphysis; ey, eye; fb, forebrain; fp, floorplate; hb, hindbrain; mb, midbrain; mn, motor axons; ob, olfactory bulb; Oto, otocyst; sc, spinal cord; ys, yolk sack. (A-H) Confocal images; (A-F) dorsal view, rostral at the top; (G) ventral view, rostral at the top; (H) lateral view, rostral towards the left. (I) Photomicrograph of a cryosection, rostral at the top. Scale bars: 100 μm in A; 10 μm in B; 50 μm in C-F; 5 μm in G; 10 μm in H; 50 μm in I.

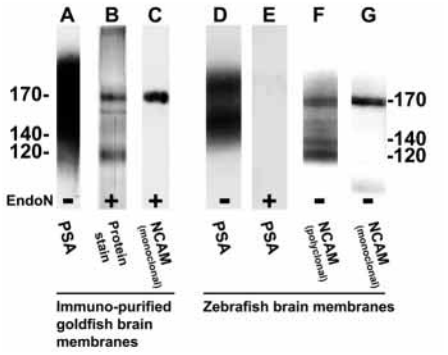

Fig. 2. Characterisation of PSA glycoproteins in goldfish and zebrafish brain. Western blots were reacted with antibodies against PSA or against NCAM. (A-C) Proteins from goldfish brain membranes immunoprecipitated with mAb 735 against PSA. They consist of a broad high molecular weight smear between 120 and 240 kDa when reacted with mAb 735 (A). After endo N treatment, discrete bands are visible at 120 kDa and 170 kDa in a protein stain (B). The band at 170 kDa is also recognised by mAb D3 against the intracellular domain of chick NCAM (C). (D-G) Immunoblots of zebrafish brain membranes reacted with mAb 735 (D,E), a polyclonal serum against goldfish proteins immunoprecipitated with mAb 735 (F) and mAb D3 (G). mAb 735 recognised a broad band between 120 and 240 kDa (D). This labeling was completely abolished when the membranes were treated with endo N before electrophoresis (E). The polyclonal serum recognised three major bands at 170 kDa, 140 kDa and 120 kDa (F), a pattern typical of NCAM in higher vertebrates. mAb D3 recognised a band at 170 kDa (G).

at 18 hpf in the spinal cord and between 20-22 hpf in the hindbrain. It is still visible in whole-mounts of 5 day old embryos (not shown). By 48 hpf, PSA levels begin to decrease and cryosections of 13-day-old zebrafish larvae show that PSA is absent in most parts of the CNS, except for a weak expression on a few cells close to the ventricle and along the pial surface (Fig. 1I, arrowheads) and a strong expression along the midline (Fig. 1I, arrow). In summary, PSA in the zebrafish CNS is almost exclusively expressed on cell bodies and, except for the posterior commissure, is not found in axonal tracts.

PSA is associated with zebrafish NCAM

To establish that PSA is associated with NCAM in fish, as it is in other vertebrates, we immunoprecipitated proteins from goldfish brain membranes with anti-PSA mAb 735. We used goldfish brains because large quantities of tissue can be obtained more easily than they can with zebrafish brains. Proteins isolated with mAb 735 were separated by SDS-gel electrophoresis and western blotted. This produced a broad band between 120 and 240 kDa when reacted with mAb 735 (Fig. 2A). After endo N treatment, two discrete bands at 120 kDa and 170 kDa were distinguishable with a protein stain (Fig. 2B). The band at 170 kDa is also recognised by mAb D3 (Fig. 2C), an antibody against the intracellular domain of chick NCAM (Schlosshauer, 1989). This antibody also crossreacts with the fish protein (Bastmeyer et al., 1990), indicating that the immunopurified proteins contain NCAM.

A polyclonal antibody that crossreacts with fish NCAM is not, to our knowledge, available. We therefore immunised a rabbit with the immunoprecipitated and endo N-treated mAb 735 proteins. The serum obtained was used for immunoblot analysis. On zebrafish brain membranes separated by SDS-gel electrophoresis and immunoblotted, mAb 735 recognised a broad band between 120 and 240 kDa (Fig. 2D). This labeling was completely abolished when the membranes were treated with endo N before electrophoresis (Fig. 2E). Our polyclonal serum recognised major bands at 170 kDa, 140 kDa and 120 kDa (Fig. 2F), a pattern typical of NCAM in higher vertebrates. On adjacent lanes of brain membranes, mAb D3 recognised a band at 170 kDa (Fig. 2G), as previously reported for goldfish brain (Bastmeyer et al., 1990). Together, these results indicate that, as in other vertebrates, PSA in zebrafish and goldfish is associated with NCAM.

Removal of PSA by endo N affects the growth pattern of the posterior commissure

To study the function of PSA during zebrafish CNS development, endo N was injected into the ventricle of 20 hpf embryos. Control embryos received an injection of buffer only. Immunocytochemistry revealed that a single injection of endo N was sufficient to remove PSA for at least 24 hours (Fig. 3A,B). After endo N injection at 20 hpf, the embryos appeared to develop normally and the overall appearance of the major axon tracts was not altered (Fig. 3C). The temporal development of the embryo is not slowed by PSA removal. The lateral line nerve reaches the same caudal spinal segments as in controls and the outgrowth pattern of secondary motoneurones, as determined by the most caudal somite with visible secondary motor axons (Ott et al., 2001), is also not delayed in the endo N-treated fish.

By contrast, the projection pattern of specific commissural axon populations was affected. Whereas most commissures in the forebrain, including the anterior commissure and the postoptic commissure, appear normal in endo N-treated

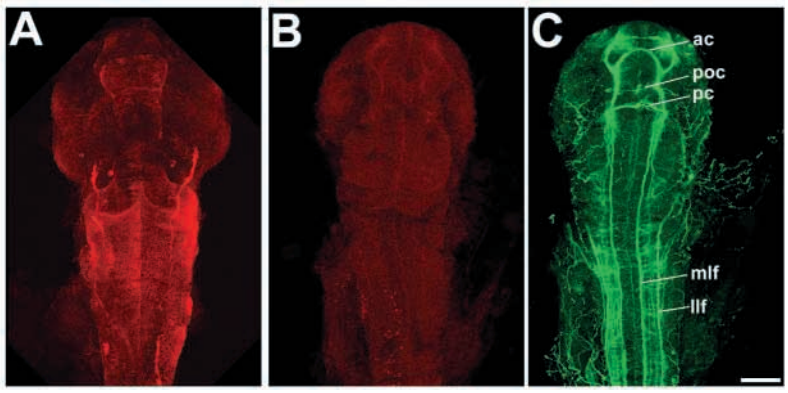

Fig. 3. A single injection of endo N removes PSA for at least 24 hours. (A) PSA staining in 32 hpf zebrafish embryo. (B,C) Zebrafish embryo at 32 hpf that received an injection of Endo N into the ventricle at 20 hpf, double labelled with PSA and acetylated α-tubulin antibodies. (B) The specific PSA labeling has disappeared and only a faint overall fluorescence remains. (C) The tubulin label demonstrates that most longitudinal and commissural axon tracts appear normal, indicating that PSA removal does not interfere with overall development of the zebrafish nervous system. ac, anterior commissure; llf, lateral longitudinal fascicle; mlf, medial longitudinal fascicle; pc, posterior commissure; poc, postoptic commissure. (A-C) Confocal images, dorsal view, rostral at the top. Scale bar: 50 μm.

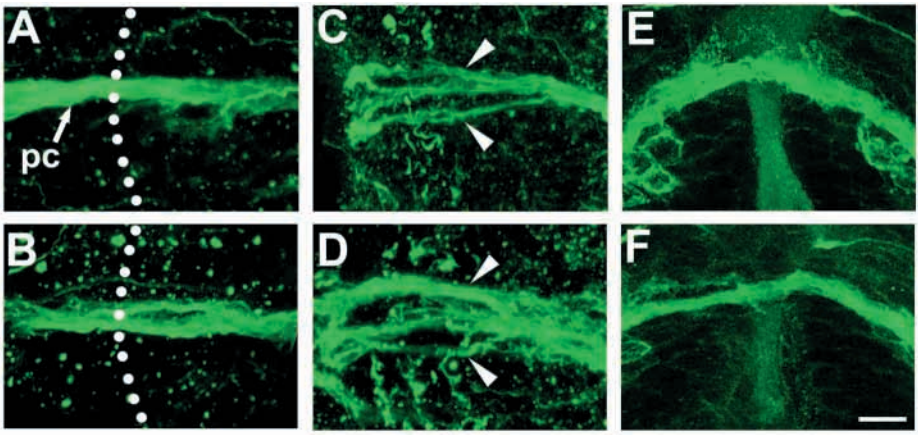

Fig. 4. Removal of PSA causes a defasciculation in the posterior commissure. (A-F) The posterior commissure (pc) in zebrafish embryos at 32 hpf after injections into the ventricle at 20 hpf, labelled with acetylated α-tubulin antibodies. (A,B) In buffer-injected control embryos the pc crosses the midline (dotted line) in one thick bundle. (C,D) In endo N-injected embryos, axons of the pc split into several thin bundles that cross the midline (arrowheads). (E,F) In PSA-polymer injected embryos, the fasciculated growth pattern of the pc is not affected. (A-F) All images are confocal images, dorsal view, rostral at the top. Scale bar: 10 μm.

embryos, PSA-positive axons in the posterior commissure (pc) were affected. Axons of the pc originate from several clusters of neurones located in lateral aspects of the rostral midbrain (Chitnis and Kuwada, 1990). They extend dorsally and cross the dorsal midline caudal to the epiphysis. The pc is pioneered by a few axons between 20-22 hpf and contains about 1700 axons at 48 hpf (Wilson et al., 1990). At the time of the initial projection, the pc axons are highly PSA positive but grow in an environment of cells that express lower levels of PSA (Fig. 1E,F). In buffer-injected embryos (n=28) labelled with anti-tubulin antibody at 32 hpf, the pc appeared as a fasciculated bundle (Fig. 4A,B). After endo N treatment, axons were defasciculated in more than 50% of the embryos (n=33), so that axons crossed the midline in several, smaller bundles (Fig. 4C,D). To quantify this effect, we counted the number of axon bundles crossing the midline in embryos from one experiment. Whereas 1.9±0.7 (n=15) bundles crossed the midline in buffer-injected embryos, there were 4.9±1.4 (n=15) bundles in endo N-treated embryos.

Injection of soluble PSA does not alter the growth pattern of the posterior commissure

The effect of soluble PSA (colominic acid, chain length 30-40 residues) can be used to help determine whether the action of membrane-associated PSA-NCAM reflects a negative effect on cell interaction via a steric mechanism at the cell surface, or a type of positive action similar to its binding of proteoglycans or growth factors. In the case of the posterior commissure described above, the injection of soluble PSA did not affect the growth pattern (Fig. 4E,F), which appears normal in all injected embryos (n=42). In control embryos that received an injection of a trimeric sialic acid chain, the growth pattern was also not altered (n=28, not shown). This suggests that PSA expression in this context is exerting a negative, steric regulation of cell interactions, as has been proposed to explain the behaviour of PSA-positive motor axons the plexus region of the chick hindlimb (Tang et al., 1994).

Removal of PSA affects midline crossing by commissural axons in the hindbrain

The second axonal projections affected by endo N are PSA-negative hindbrain commissures. Hindbrain commissural axons originate from reticulospinal neurones (Mendelson, 1986; Metcalfe et al., 1986), follow a characteristically curved pathway and project into the contralateral medial longitudinal fascicle (mlf). In the zebrafish hindbrain, the first commissural axons cross the midline between 18-20 hpf (Weiland et al., 1997), before PSA is upregulated on floorplate cells. From 22 hpf onwards, at which time PSA is upregulated on floorplate cells, other commissural axons become visible, following a straight path across the midline (Weiland et al., 1997). These belong either to a group of axons that project rostrally via the mlf into the contralateral midbrain (Trevarrov et al., 1990) or are from commissural interneurones that project into the contralateral hindbrain segment. With further development, more commissural axons are added and form several bundles in each hindbrain segment. These bundles are negative for PSA (Fig. 5A,C) but can be visualised with anti-Tag-1 antibodies or with mAb 6-11B-1 against acetylated α-tubulin (Fig. 5B,D). They cross the mlf and upon contact with the PSA-positive floorplate cells (Fig. 5C), defasciculate into single axons that cross the midline (Fig. 5D, arrowheads). This regular growth pattern was observed in all of the buffer-injected control embryos (n=56), but was markedly disturbed after enzymatic PSA removal in about 50% of endo N-injected embryos (n=60). That is, bundles of axons still grew ventrally and crossed the mlf, but fewer axons crossed the midline (Fig. 5E) and appeared to stop at the PSA-negative floorplate cells (Fig. 5F, arrowheads).

As mAb 6-11B-1 against acetylated α-tubulin labels almost all axons at that developmental stage, the behaviour of specific commissural axon populations might be obscured in whole-mount stain. We therefore used DiI as an anterograde tracer in combination with endo N treatment to analyse the growth pattern of specific axons in more detail. A small crystal of DiI was inserted into the hindbrain just rostral to the otocyst in embryos fixed at 36 hpf (Fig. 6C). DiI crystals in deeper positions labelled axons that project into the ipsilateral and contralateral mlf and then course rostrally or caudally (not shown). DiI crystals in most dorsal positions labelled a small number of axons that extend ventrally. These axons cross the ipsilateral mlf, the midline and the contralateral mlf and terminate in mid-dorsal positions on the contralateral side of the same hindbrain segment (Fig. 6C-F), indicating that they belong to commissural interneurones. In 98% of control-injected embryos with effective DiI placements (n=46), all labelled axons crossed the midline and terminated without

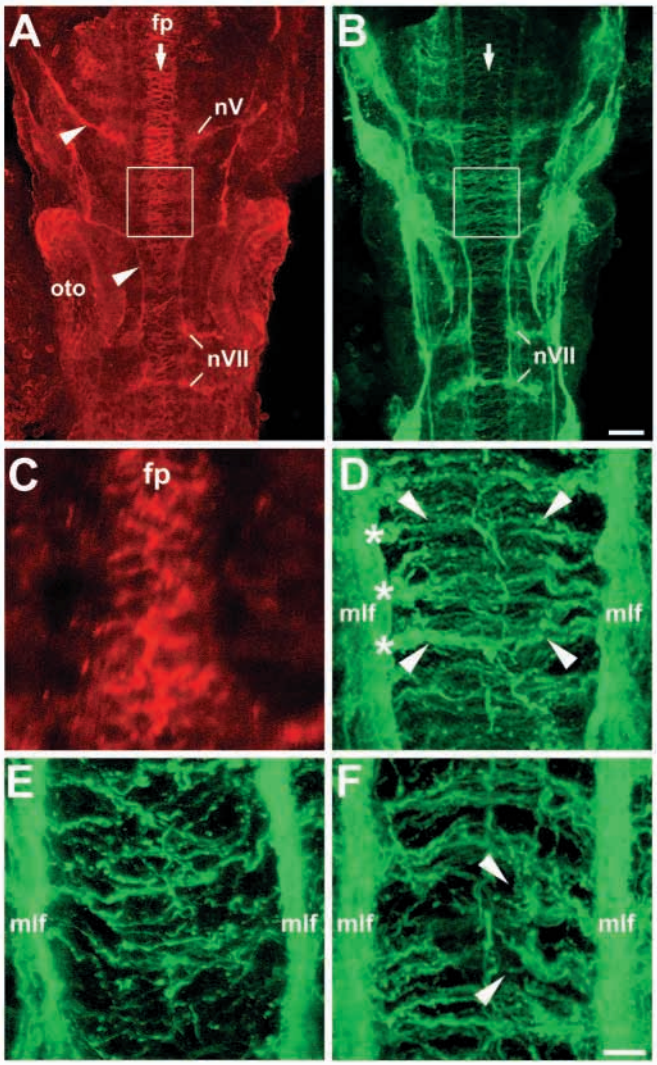

Fig. 5. Removal of PSA interferes with the development of hindbrain commissures. (A,B) Hindbrain of a buffer-injected control embryo at 32 hpf labelled with antibodies against PSA and Tag-1. (A) PSA is expressed by the motor nuclei of cranial nerves (nV and nVII), by their motor axons leaving the CNS (arrowheads) and on cells of the floorplate (fp). (B) Tag-1 labeling shows the VII cranial nerve and commissural axons crossing the midline (B, arrow). The boxed regions rostral to the otocyst (oto) in A and B represent the position chosen for the analysis of commissural axon growth. (C) In control embryos, PSA is expressed on cells of the floorplate (fp) but not on commissural axons. (D) Tubulin labeling of the same embryo shows commissural axons crossing the medial longitudinal fascicle (mlf) in thick bundles (asterisks). These axons defasciculate (arrowheads in D) upon arrival at the PSA-positive floorplate cells. (E,F) The growth pattern of commissural axons is markedly disturbed in endo N-injected embryos. Fewer axons cross the floorplate (E) or bundles of commissural axons appear to stop at the PSA-negative floorplate cells (arrowheads in F). All images are confocal images, ventral views, rostral at the top. Scale bars: in B, 20 μm in A,B; in F, 5 μm in C-F.

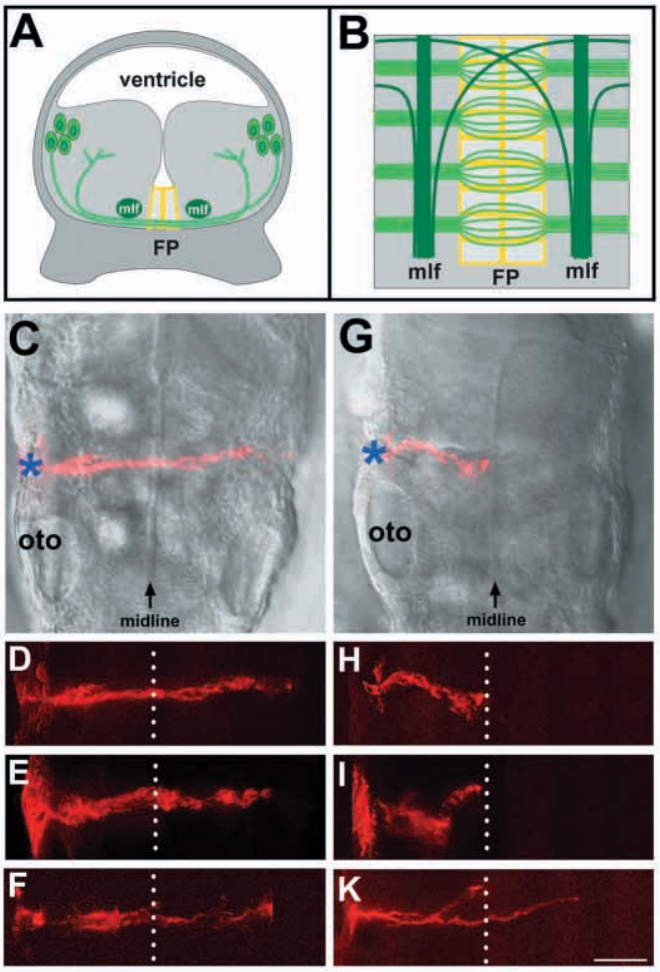

Fig. 6. Removal of PSA prevents commissural axons in the hindbrain from crossing the midline. Schematic drawings illustrate the projection pattern of commissural axons in the hindbrain in cross section (A) and in a ventral view (B). Cell bodies of commissural interneurones occupy a dorsolateral position in each hindbrain segment. Their axons extend ventrally, cross the medial longitudinal fascicle (mlf) and the floorplate (FP) and terminate in contralateral mid-dorsal positions in the same hindbrain segment (A). Axons of reticulospinal neurones (B, dark green) cross the floorplate in a curved pathway and project into the contralateral mlf. Axons of commissural interneurones (B, light green) cross the mlf in a straight pathway and defasciculate upon contact with PSA-positive floorplate cells (yellow). (C,G) Hindbrain of zebrafish embryos at 36 hpf in an overlay of interference contrast and DiI-fluorescence. To analyse the growth pattern of commissural axons in more detail, DiI was inserted close to the cell bodies of commissural interneurones (asterisks) rostral to the otocyst (oto). (C-F) In buffer-injected control embryos, all DiI-labelled axons cross the midline (dotted line) and terminate on the contralateral side. (G-K) In endo N-injected embryos, either all (H,I) or subsets (K) of DiI-labelled axons stop at the midline. (C-K) Dorsal views, rostral at the top. Scale bar: 50 μm.

bifurcating on the contralateral side (Fig. 7C). By contrast, abnormalities in the growth pattern of DiI-labelled commissural axons were observed in 54% of endo N-injected embryos (n=72) (Fig. 7C). These abnormalities consisted of situations in which either all DiI-labelled axons (Fig. 6G-I), or subsets of axons (Fig. 6K), stopped close to the midline. The nature of this altered behaviour suggests that PSA expression by floorplate cells facilitates midline crossing of commissural axons in the hindbrain.

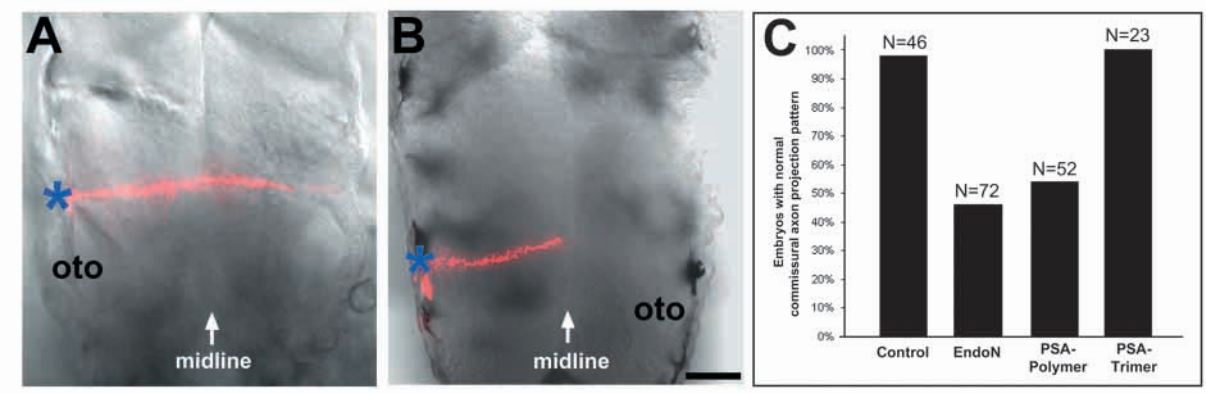

Fig. 7. Injection of soluble PSA causes the same defects in the growth pattern of commissural axons in the hindbrain as does removal of PSA. (A,B) Hindbrain of zebrafish embryos at 36 hpf in an overlay of interference contrast and DiI fluorescence (asterisks mark DiI injection sites). (A) In sialic acid trimer-injected embryos, DiI-labelled commissural axons cross the midline and reach the contralateral side. (B) Hindbrain of a soluble-PSA injected embryo. The commissural axons fail to reach the other side and stop at the midline. oto, otocyst. Scale bar: 50 μm. (C) Quantification of the effects on the growth pattern of hindbrain commissural axons caused by the injection of control-buffer, endo N, PSA-polymer and PSA-trimer.

Injection of soluble PSA affects midline crossing of commissural axons in the hindbrain

As above for the pc axons, we injected soluble PSA (colominic acid) or a control solution (sialic acid trimer) into zebrafish embryos at 20 hpf. Both groups of embryos were fixed at 36 hpf and analysed by DiI labeling. In all of the sialic acid trimer-injected embryos (n=23), the commissural axons appeared normal (Fig. 7A,C). However, 46% of the colominic acid-injected embryos (n=52) exhibited abnormalities in hindbrain commissures, namely axons that stopped close to the midline (Fig. 7B,C). This suggests that in hindbrain commissures, where PSA-negative axons are in a PSA-positive environment, PSA functions in a way that is distinct from that observed for the highly PSA-positive posterior commissure, where axons are in an environment of lower PSA levels.

DISCUSSION

The results obtained in this study reveal three novel aspects of PSA function. First, the developing zebrafish CNS displays a pattern of PSA-NCAM expression that is strikingly different from that found in other vertebrates. Second, PSA affects the growth behaviour of two types of commissural axons: one involving fasciculation, which appears similar to observations made for CNS axons in other vertebrates; and one involving midline crossing that has not been previously observed. Finally, our studies suggest that the role of PSA in midline crossing involves a molecular mechanism that is distinct from that believed to operate in other axon outgrowth systems. In the following discussion, the importance of these findings is evaluated in the context of the extensive literature on PSA in chicken and mouse embryos.

The expression of PSA in the developing CNS of mouse or chick is dominated by the presence of high levels of heavily polysialylated NCAM on axonal processes in nearly every tract (Chuong and Edelman, 1984). As a result, immunohistochemistry for PSA in the brain of these species results in an almost uninterpretable mass of staining throughout much of the tissue. The selective expression of PSA on a small set of distinct tracts in the zebrafish was therefore a surprise, and indicates that the role of PSA in fish is probably different in scope and perhaps nature, when compared with avian and mammalian vertebrates. A more limited expression of PSA on zebrafish motoneurones, namely an absence on primary axons but heavy labelling of secondary axons (this study; U. R. and J. Eisen, unpublished), also supports such a phylogenetic difference in PSA use.

In contrast to axon tracts, CNS cell bodies in the zebrafish display PSA quite broadly. PSA expression on neural precursors has been widely documented (Miragall, 1988; Murakami et al., 1991) and is, in some cases, related to the ability of these cells to migrate from their site of origin to distinct parts of the brain (Ono et al., 1994; Wang et al., 1994; Hu et al., 1996). Although we did not investigate cell migration in this study, it is possible that PSA function on neural precursors is more similar in vertebrates than its effect on the subsequent elaboration of axonal processes.

In all previous studies on axonal tracts of other vertebrates, PSA was found to influence either the fasciculation pattern of axon bundles (Tang et al., 1992; Yin et al., 1995; Honig and Rutishauser, 1996) or the formation of collateral branches (Daston et al., 1996), but had little or no effect on axonal elongation. Although the contexts differed widely, in each case PSA could be viewed as a steric negative regulator of membrane-membrane apposition, which serves to attenuate a variety of cell-cell interactions (Fig. 8A) (Rutishauser, 1998). This mechanism requires that the PSA be membrane bound and thus is eliminated by exposure of the cells to endo N but is unaffected by injection of soluble PSA. In the case of axons, the reduced interaction produced by expression of cell-surface PSA behaves as a permissive factor that promotes either the dispersal of a fascicle by loosening axon-axon adhesions (Tang et al., 1994) (Fig. 8B), or conversely the growth of axons along other axons by shielding the axon from even stronger adhesive influences such as those that lead to synapse formation (Seki and Rutishauser, 1998) (Fig. 8B). Which of

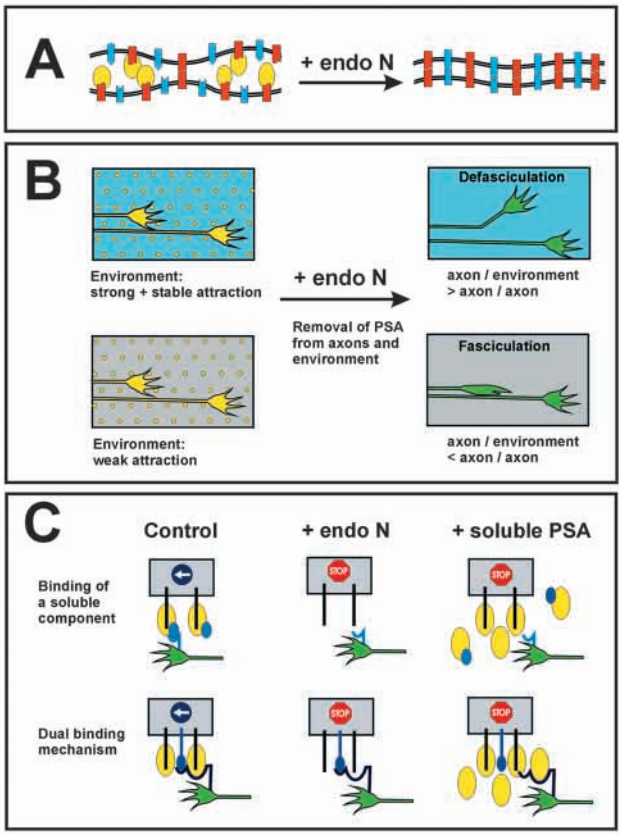

Fig. 8. Effects of PSA on cell interactions and axon fasciculation. (A) When PSA (yellow) is attached to the cell surface via NCAM, its steric properties hinder the apposition of cell membranes and therefore reduce the interaction of receptors, both NCAM (orange) and non-NCAM (blue), on apposing cells. When PSA is removed, receptors can form enough cell-cell bonds for stable adhesion to occur. This steric effect cannot be blocked by soluble PSA. (B) Removal of PSA from the axons and/or their environment can have opposite effects on PSA-positive axon tracts, depending on the nature of the environment. Top: if the environment has powerful and stable adhesive attractants, such as potential sites for junctions (Seki and Rutishauser, 1998), loss of PSA can induce axons to follow more independent, less fasciculated pathways. Bottom: if the environment does not support such stable interactions, as in the matrix-rich plexus region of the chick hindlimb (Tang et al., 1994), the intrinsic interactions among the axons will prevail and loss of PSA will tend to promote growth of axons along other axons to form larger fascicles. In the present study, the PSA-positive posterior commissure, which traverses an environment that contains cells that are also PSA-positive, is shown to defasciculate partially when PSA is removed; this effect is not mimicked by soluble PSA. (C) Two possible binding-based modes for PSA action during midline crossing of commissural axons in the hindbrain. Top: PSA (yellow circles) binds a secreted component (blue circles) necessary for midline crossing, thereby keeping this factor at a high concentration on the surface of floorplate cells. Bottom: a dual binding mechanism in which PSA and a second molecule (blue) on the floorplate cell combine to bind to and activate an axonal receptor. Unlike steric models for PSA action (A,B), the binding-based mechanisms are predicted to be competitively inhibited by soluble PSA and blocked by endo N treatment.

these two behaviours occurs depends primarily on two variables: the presence of PSA on the axons and/or the environment, and the nature and susceptibility to PSA of cell-cell interactions offered to the axons by that environment.

The perturbation of PSA function in zebrafish by specific in vivo enzymatic degradation of the carbohydrate homopolymer with endo N has yielded both expected and unexpected results. The defasciculation of the posterior commissure produced by endo N (but not soluble PSA), without noticeably affecting the number of fibres that cross this terrain, most closely resembles effects reported for optic axons in the chick tectum (Yin et al., 1995) and mossy fibres in the mouse hippocampus (Cremer et al., 1997; Seki and Rutishauser, 1998). As in those contexts, PSA is expressed both by the commissural axons and by the surrounding CNS environment, and its removal would appear to increase environmental interactions that attract growth cones into paths not normally taken by these axons (Fig. 8B). Of course, axon-axon interactions should also be increased, but in the absence of PSA appear to be less potent than those offered to the axons by the environment. In the hippocampus, for example, it has been suggested that without PSA the typical IgCAM-mediated adhesion between axons in a fascicle (Tang et al., 1994), appears unable to compete effectively with the more stable association of individual axons with cadherin-associated junctions (Seki and Rutishauser, 1998).

By contrast, the inability of specific hindbrain commissural axons to cross the midline is a new phenomenon for endo N-treated embryos and is clearly distinct from the behaviour induced in the posterior commissure. Axon guidance at the midline choice point is a complex process that requires a variety of molecules (Stoeckli, 1998; Kaprielian et al., 2001). Midline floorplate cells do express both attractive and repulsive components (Stoeckli et al., 1997) that interact with axonal receptors to regulate the growth pattern of ipsi- and contratateral projections. In the zebrafish hindbrain, commissural axons of interneurones are affected upon PSA removal, whereas reticulospinal axons, which cross the midline before PSA is upregulated, are not affected. Whether commissural axons in the zebrafish spinal cord were also affected after PSA removal could not be analysed in this study because of technical difficulties. Hindbrain commissural axons, like most tracts in the developing zebrafish, do not express PSA. Floorplate cells, however, do express PSA and thus are the likely source of the perturbation. In the mouse spinal cord, both commissural axons and floorplate cells express PSA (Boisseau et al., 1991). Whether the removal of PSA also affects commissural axon growth in chick and mice has not been reported.

Remarkably, injection of endo N induced an abrupt cessation of axon elongation at the midline that was anatomically indistinguishable from that produced by soluble PSA. As indicated in the Introduction, such an effect is indicative of a very different role for PSA, namely that of promoting rather than inhibiting interaction between cells. Fig. 8 illustrates two non-steric modes by which PSA could in principle act during midline crossing of commissural axons in the hindbrain. In the first mode (Fig. 8C), PSA binds a secreted component necessary for midline crossing, thus keeping it at a high concentration at the surface of floorplate cells. Removal of PSA would therefore reduce the local concentration of this compound and hinder crossing. In the

second mode (Fig. 8C), PSA combines with a receptor on floorplate cells in order to bind to and trigger an second axon-associated receptor that permits midline crossing by that axon.

There is some precedent for each of these binding-based mechanisms. For example, binding of brain-derived neurotrophic factor (BDNF) has been proposed to be a mechanism by which PSA can influence long-term potentiation in the hippocampus (Muller et al., 2000). However, PSA appears to be a second binding component in the interaction of NCAM-expressing cells with heparan sulphate proteoglycans (Storms and Rutishauser, 1998). Although the available data are not sufficient to choose between these two possibilities, manipulation of these new potential binding partners should be useful in future studies.

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