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Polysialyltransferase Expression Is Linked to Neuronal Migration in the Developing and Adult Zebrafish

Sandra Rieger, Katrin Volkmann, and Reinhard W. Köster*

Modulation of cell–cell adhesion is crucial for regulating neuronal migration and maintenance of structural plasticity in the embryonic and mature brain. Such modulation can be obtained by the enzymatic attachment of polysialic acid (PSA) to the neural cell adhesion molecule (NCAM) by means of the polysialyltransferases STX and PST. Thus, differential expression of STX and PST is likely to be responsible for varying functions of PSA-NCAM during neuronal differentiation, maintenance, plasticity, and regeneration. We have isolated the zebrafish homologues of STX (St8sia2) and PST (St8sia4) and demonstrate that their expression in the embryonic and adult nervous system is often confined to regions of neuronal migration. Moreover, in the adult cerebellum, the complementary expression pattern of both polysialyltransferases suggests a function in regulating cerebellar neuronal plasticity. Enzymatic removal of PSA in the embryonic cerebellum results in impaired neuronal migration, suggesting that PSA-NCAM is a key regulator of motility for cerebellar neuronal progenitors. ***Developmental Dynamics 237:276–285, 2008.***

Key words: polysialyltransferase; PSA-NCAM; cell adhesion; zebrafish; cerebellum; neuronal migration; bio-imaging

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INTRODUCTION

The neural cell adhesion molecule (NCAM) plays multiple roles during the development of the central nervous system (CNS). It has been shown to be involved in regulating embryonic neurogenesis (Ponti et al., 2006), neuronal migration (Ono et al., 1994; Wang et al., 1994; Hu et al., 1996), axonal pathfinding (Tang et al., 1992; Daston et al., 1996; Cremer et al., 1997; Marx et al., 2001), as well as synaptogenesis (Seki and Rutishauser, 1998). Furthermore, in the mature brain, NCAM has been implicated in regulating adult neurogenesis (Vutskits et al., 2006), neuronal migration (Cremer et al., 1994), survival of neural stem cell derived neuronal progenitors (Gascon et al., 2007), synaptic plasticity, and learning (Becker et al., 1996; Muller et al., 1996). During these processes the properties of NCAM are modulated by the reversible attachment of polysialic acid (PSA) to its extracellular domain. For example, PSA weakens the adhesive properties of NCAM by steric hindrance and thus promotes migration of neuronal progenitors in the rostral migratory stream of the vertebrate olfactory system (Ono et al., 1994; Rutishauser and Landmesser, 1996; Rutishauser, 1998). In addition, PSA-bearing NCAM regulates the expression of the low-affinity neurotrophin receptor p75 and thereby promotes survival of these migrating neuronal progenitors (Gascon et al., 2007). PSA attached to NCAM has also been involved in binding and presenting growth factors thereby regulating long-term potentiation (Becker et al., 1996; Muller et al., 2000). Therefore, reversible PSA attachment

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GSF- National Research Center for Environment and Health, Institute of Developmental Genetics, Neuherberg-Munich, Germany

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***Correspondence to: Reinhard W. Köster, GSF- National Research Center for Environment and Health, Institute of Developmental Genetics, Ingolstädter Landstrasse 1, 85764 Neuherberg-Munich, Germany. E-mail: reinhard.koester@gsf.de**

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and removal is crucial for modulating NCAM-activity and, thus, CNS development and function.

PSA synthesis on NCAM is specifically mediated by two polysialyltransferases, STX and PST, which act in a differential as well as synergistic manner (Ong et al., 1998; Angata et al., 1998; Nakayama et al., 1998; Angata and Fukuda, 2003; Angata et al., 2004; Weinhold et al., 2005). Based on their differential expression PSA synthesis during embryogenesis has mainly been attributed to STX, whereas adult PSA synthesis is thought to be mediated preferentially by PST (Becker et al., 1996; Hildebrandt et al., 1998; Ong et al., 1998). The expression and function of these polysialyltransferases has attracted increasing biomedical interest for several reasons: (1) high PSA levels as well as STX expression can serve as diagnostic markers for neuroblastomas with poor prognostic outcome (Cheung et al., 2006; Seidenfaden et al., 2003); (2) polysialyltransferase activity promotes metastasis formation and, thus, represents an interesting pharmaceutical target; (3) the migration promoting activity of either STX- or PST-mediated PSA synthesis in genetically engineered Schwann cells significantly supports regeneration by increasing axon outgrowth and Schwann cell–mediated remyelination (Lavdas et al., 2006; Zhang et al., 2007).

Due to their small size, transparency, and extracorporal development, zebrafish embryos allow one to observe highly dynamic cell behaviors such as neuronal migration (Köster and Fraser, 2006), axon pathfinding (Fricke et al., 2001), or synaptogenesis (Niell et al., 2004; Mumm et al., 2006) directly in the living organism and are, thus, well suited to address PSA-NCAM and polysialyltransferase function in vivo. Moreover, in contrast to mammals, adult zebrafish show significant levels of neurogenesis in almost all regions of the adult brain and have a pronounced capability to regenerate CNS lesions (Becker et al., 2004; Zupanc et al., 2005; Zupanc and Zupanc, 2006; Adolf et al., 2006; Grandel et al., 2006). For example, stab wounds in the adult zebrafish cerebellum are repaired within several days by an up-regulation of neurogenesis in the cerebellar molecular layer followed by directed migration of these neuronal progenitors toward the lesion site (Liu et al., 2004; Zupanc and Zupanc, 2006). Such adult neurogenesis, migration, and regeneration in zebrafish could likely involve PSA-NCAM function, but research on PSA-NCAM has only been initiated recently. In an elegant study, polysialylation of NCAM in zebrafish embryos has been demonstrated to play a crucial role in axon fasciculation, but also commissural axonal guidance during midline crossing (Marx et al., 2001). Recently, the cloning and expression of the highly conserved zebrafish STX- and PST-homologues were reported and both were shown to be capable of synthesizing PSA (Marx et al., 2007). Consistent with its predominant expression during embryogenesis, antisense-morpholino studies revealed that STX is likely to be solely responsible for PSA synthesis during zebrafish embryogenesis. This finding is in good agreement with mammalian STX and PST function (Eckhardt et al., 2000; Angata et al., 2004). Surprisingly though, in the adult zebrafish brain, only STX expression was found to correlate with PSA synthesis, while PST expression was either weak or absent, suggesting that STX may be the sole PSA-synthesizing enzyme relevant in the developing and adult zebrafish CNS (Marx et al., 2007).

In addition to axonal fasciculation and guidance, additional functional roles for PSA-NCAM during zebrafish embryogenesis remained unclear so far. To address whether PSA-NCAM in zebrafish is involved in regulating neuronal migration, we have independently isolated zebrafish homologues of the polysialyltransferases STX and PST and compared their expression to NCAM and PSA. Intriguingly, in the developing cerebellum, where extensive neuronal migration from the cerebellar rhombic lip has been shown (Köster and Fraser, 2001a), NCAM is broadly expressed, while STX and PSA expression delineate the anteroventral pathway of cerebellar rhombic lip derived neuronal progenitors during early migration. Furthermore, in vivo time-lapse analysis reveals that neuronal migration from the cerebellar rhombic lip is inhibited after enzymatic removal of PSA, suggesting a migration-promoting function for the polysialylation of NCAM. Moreover, in contrast to the previous report, we find extensive expression for PST in numerous regions throughout the adult CNS. In particular in the adult cerebellum, PST and STX show an overlapping but complementary expression pattern. While STX is strongly expressed in the Purkinje cell layer, PST shows stronger expression in the granule cell layer. This finding suggests that, in the adult zebrafish cerebellum, cell type specific regulation of PSA synthesis is mediated by cooperative and differential polysialyltransferase expression in differentiated neurons and, thus, may serve additional functions such as regulating the plasticity of cerebellar neuronal circuitries. Thus, our study suggests that PST and STX functions are much more conserved between teleosts and mammals than previously thought and lay the ground for PSA-NCAM functional studies on adult CNS regeneration in vertebrates.

RESULTS

Polysialyltransferase Expression in the Developing Zebrafish CNS

Polysialylated NCAM has been implicated in the control of neuronal migration during mammalian nervous system development (Murakami et al., 2000; Marx et al., 2001; Ulfig and Chan, 2004). The two polysialyltransferases STX and PST are key enzymes in the synthesis of polysialic acid on NCAM in vertebrates (Angata and Fukuda, 2003). Zebrafish homologues of *pst* and *stx* were initially identified by sequence similarity searches in the zebrafish genome (Harduin-Lepers et al., 2005). Recently, the cloning of these homologues by reverse transcriptase-polymerase chain reaction (RT-PCR) and their first functional characterization in axon pathfinding has been reported (Marx et al., 2007). To address a possible role for polysialyltransferase activity during neuronal migration in the developing and mature zebrafish nervous system, we have independently cloned both polysialyltransferases from cDNA of 54 hpf zebrafish embryos. The sequences

of both genes correspond to those reported previously (Harduin-Lepers et al., 2005; Marx et al., 2007). The onset of *stx* expression had been determined to occur at approximately 13 hours postfertilization (hpf) and to remain relatively ubiquitously in the nervous system until approximately 40 hpf, when expression was found to decline in most regions (Marx et al., 2007). We focused our analyses on differentiation stages of CNS development and found, consistent with Marx et al., that continued high *stx* expression at 48 hpf remains along the dorsal hindbrain brain ventricle (Fig. 1A, black arrowhead), in the differentiating cerebellum (Fig. 1A), and in some cranial motor neurons coexpressing GFP in embryos of the transgenic islet1:GFP line (Fig. 1G–J, white asterisks; Higashijima et al., 2000), all are domains of pronounced neuronal migration. Low *stx* expression levels were detected in the telencephalon, diencephalon, tectum, and the anterior spinal cord. At 96 hpf when migration ceases in the zebrafish nervous system, *stx* expression levels declined in these regions and only faint expression remained in the cerebellum (Fig. 1B) and along the ventricles (not shown). At 7 dpf, weak *stx* staining was retained in a dorsomedial domain of the anterior hindbrain (Fig. 1C dashed box and inset), while *stx* expression was not evident in other brain regions anymore.

Expression of the polysialyltransferase *pst* was generally weaker in the CNS throughout development when compared with *stx* (Fig. 1D–F). At 48 hpf, *pst* expression was detected in ventral regions of the fore-, mid-, and anterior hindbrain (Fig. 1D) and around the ventricles (not shown). *pst* expression was most prominent in dorsoventral stripes in the hindbrain (Fig. 1D inset, black asterisks), delineating the central domains of individual rhombomeres. Similarly to the decline in *stx* expression also *pst* levels diminished with progression of organogenesis and low expression remained in patches in the hindbrain at 96 hpf (Fig. 1E). At 1 week postfertilization, *pst* expression could not be detected further in the nervous system (Fig. 1F compare inset with Fig. 1C showing *stx* expression in this domain). These findings reveal that the spatiotemporal expression pattern of *stx* in the developing zebrafish is similar to that of mouse, where STX is the major polysialyltransferase during CNS development, but its expression declines at postnatal stages (Ong et al., 1998). In contrast, mouse PST expression is continuous and lasts at moderate levels from embryogenesis to adulthood, whereas in zebrafish PST expression is lost during juvenile stages.

STX and PST Are Both Expressed in Common and Differential Domains in the Adult Zebrafish Brain

STX and PST polysialyltransferases show moderate expression levels in varying brain regions of the adult mammalian nervous system (Angata et al., 1997; Hildebrandt et al., 1998). To compare the expression patterns of both enzymes in the adult zebrafish brain, we analyzed paraffin sections by in situ hybridization. Our results reveal that, despite the decline in the expression levels of *stx* and *pst* at 1 week post fertilization (Fig. 1C,F), expression of both enzymes reappears in the mature brain (Fig. 2A–F). Recently, PST expression was reported to be absent in the adult zebrafish nervous system, suggesting an exclusive role for STX in modulating NCAM activity in the mature brain (Marx et al., 2007). In contrast, our in situ hybridization analyses revealed a clear cell type and tissue-specific expression of *pst* in the adult brain (Fig. 2D–F).

On sagittal sections, expression of both enzyme-encoding genes were detected in the outermost glomerular layer (GL), the adjacent external cellular layer (ECL) and the internal cellular layer (ICL) of the olfactory bulb (OB; Wullimann et al., 1996), in the pallium, and in stripes of the subpallium in the telencephalon (Fig. 2B,E), which has been considered an equivalent of the rostral migratory stream (RMS) in rodents (Adolf et al., 2006). The expression domains of *stx* and *pst* in the subpallial stripe correlate well with the presence of PSA-NCAM in migrating proliferative neuroblasts (Adolf et al., 2006). Expression of both enzymes was, furthermore, detected in the periventricular gray zone of the midbrain, the optic tectum (Fig. 2A,D), and along the lateral diencephalic ventricles of the hypothalamus (Fig. 2C,F). *stx* and *pst* expression was, furthermore, found in the molecular (ML), Purkinje (PCL), and granule cell layers (GCL, white asterisks) of the corpus cerebelli and the valvula cerebelli (Fig. 2H–K), as well as in the crista cerebellaris (*pst* in Fig. 2D, not shown for *stx*). Of interest, the cells in the PCL expressed high *stx* but low *pst* levels. In contrast, cells in the GCL strongly expressed *pst* but showed weak *stx* expression (Fig. 2H–K). In addition, pronounced but unique expression of *stx* was found in the preoptic nucleus, the thalamic region (Fig. 2A), in the caudal lobe of the cerebellum (Fig. 2H, black asterisk), in the vagal lobe of the hindbrain, and in several regions of the medulla extending into the spinal cord (Fig. 2A).

Our expression analysis of *stx* and *pst* in the adult brain shows that both are not ubiquitously expressed, but that *stx* as well as *pst* expression is confined to distinct brain regions. It is noteworthy that these tissues also show expression of NCAM (e.g., corpus cerebelli shown in Fig. 2G) and PSA (not shown). These findings suggest a function for STX and PST in regulating adult neurogenesis, neuronal migration, and plasticity by means of modulation of NCAM polysialylation.

NCAM, STX, and PSA Are Coexpressed in Domains of Neuronal Migration in the Developing Zebrafish Cerebellum

Modulation of NCAM by means of PSA addition has been shown to be important for neuronal migration (Cremer et al., 1994; Ono et al., 1994; Murakami et al., 2000), but in zebrafish, such a function for PSA-NCAM remained elusive. We, therefore, compared the expression of NCAM, STX, and PSA in the differentiating cerebellum during stages of intense neuronal migration (note that *pst* is not expressed in the cerebellum during embryonic stages), when neuronal progenitors migrate from the upper rhombic lip (URL) toward and along the midbrain–hindbrain bound-

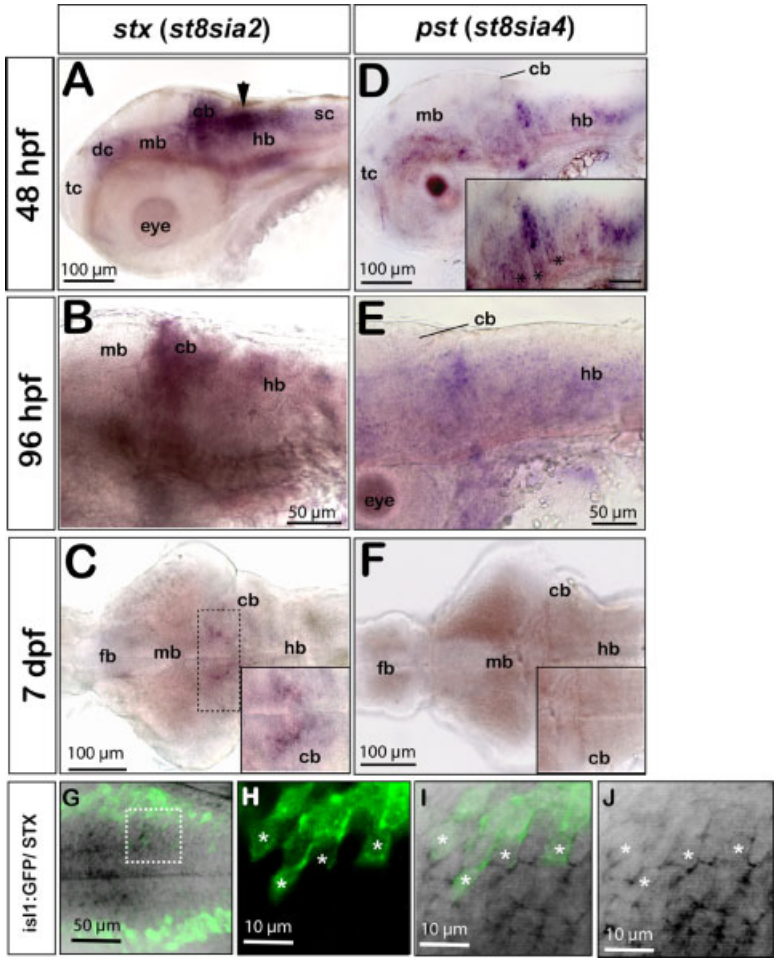

Fig. 1.

Fig. 1. *stx* and *pst* expression in the differentiating zebrafish central nervous system. Panels A,B,D,E show lateral and C,F dorsal views, anterior is to the left. **A:** At 48 hours postfertilization (hpf) *stx* expression is weakly present in the ventral telencephalon, in the diencephalon, midbrain, in the floorplate, and in the spinal cord. Strong expression is found in dorsal regions of the caudal hindbrain along the fourth ventricle (black arrow) and in the differentiating cerebellum. **B:** *stx* expression remains weakly detectable in the differentiating cerebellum and in ventral regions of the mid- and hindbrain at 96 hpf. **C:** At 7 days postfertilization (dpf), only the dorsomedial domain of the hindbrain retains low levels of *stx* (dashed box and inset showing high magnification of the dorsomedial domain in the anterior hindbrain with remaining *stx* expression). **D:** At 48 hpf, *pst* is expressed in the ventral domains of the fore- and midbrain. A striped expression pattern can be observed in the hindbrain, being confined to the central domains of individual rhombomeres (inset, asterisks). **E:** Only some patches in the hindbrain retain low *pst* expression levels at 96 hpf. **F:** *pst* expression is no longer detectable in the brain at 7 dpf (compare inset in F with inset in C). G–J: Expression of *stx* in a subset of cranial motoneurons as revealed by colabeling of *stx* and green fluorescent protein (GFP) in embryos of the islet1:GFP transgenic line. **G:** Maximum intensity projection of a 30-µm stack of optical sections showing *stx* expression in medial domains of the hindbrain (black) and more laterally positioned GFP-expressing cranial motoneurons (green). **H–J:** A subset of cranial motoneurons (H, white asterisks) expresses *stx* (J, white asterisks), which can be identified in 1-µm single optical sections at high magnification. I: Overlay of GFP and *stx* expression. cb, cerebellum; dc, diencephalon; fb, forebrain; hb, hindbrain; mb, midbrain; sc, spinal cord; tc, telencephalon

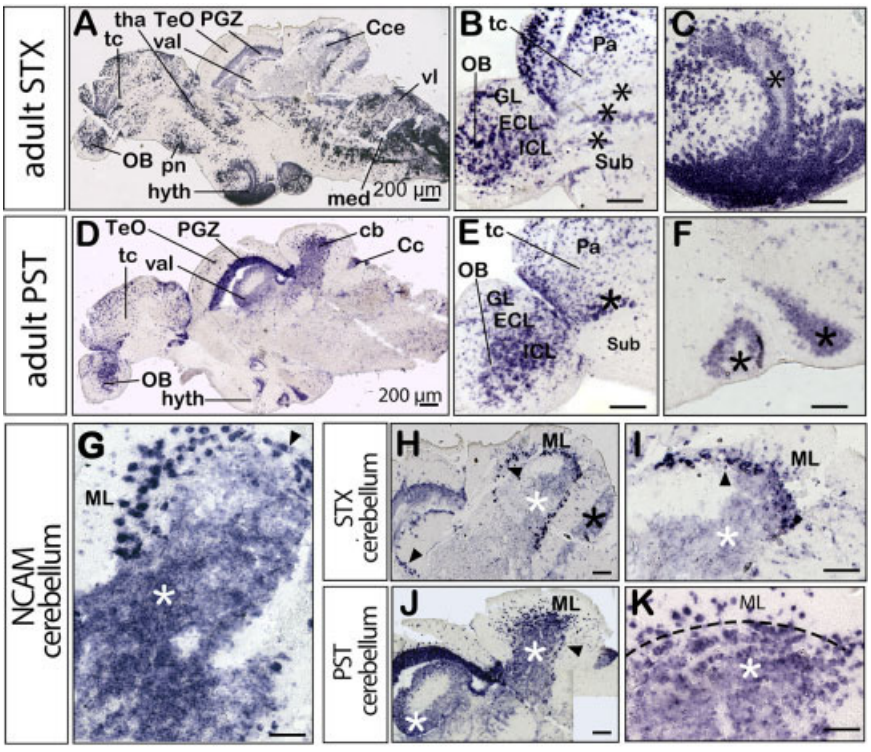

Fig. 2.

Fig. 2. Comparison of *stx* and *pst* expression in the adult zebrafish brain. All panels show medial sagittal sections, anterior is to the left. A–C,H,I show *stx* expression and D–F,J,K show *pst* expression. **A:** Overview of *stx* expression. **D:** Overview of *pst* expression. **B,E:** Cells in the pallial migratory domains of the telencephalon and olfactory bulb are marked by asterisks. **C,F:** Ventricular staining in the hypothalamic region. **G:** *ncam* expression in the molecular layer (ML), Purkinje cell layer (PCL, black arrowhead), and granule cell layer (GCL, white asterisk) of the cerebellum. **H:** Higher magnification of the cerebellum showing *stx* expression in some cells of the ML and strong expression in cells of the PCL, extending from the corpus into the valvula cerebelli (black arrowheads). Weak expression levels are present in the caudal lobe (black asterisk) and in the GCL (white asterisk). **I:** A higher magnification of *stx* expression in the adult cerebellum. **J:** Strong *pst* expression in the GCL of the corpus cerebelli and in the valvula cerebelli being more pronounced than *stx* expression (H, white asterisk). **K:** A higher magnification of *pst* expression in the adult cerebellum, being detected in the ML and GCL, separated by a dashed line. Cc, crista cerebellaris; Cce, corpus cerebelli; ECL, external cellular layer; GL, glomerular layer; hyth, hypothalamus; IL, internal cellular layer; med, medulla; ML, molecular layer; OB, olfactory bulb; Pa, pallium; PGZ, periventricular gray zone of the optic tectum (TeO); pn, preoptic nucleus; Sub, subpallium; tc, telencephalon; tha, thalamus; val, valvula cerebelli; vl, vagal lobe. Scale bars = 200 µm.

ary (MHB) to ventral brain regions (Köster and Fraser, 2001a).

Consistent with a potential role in regulating migration, uniform but strong expression of *ncam* (Fig. 3A,D) and *stx* (Fig. 3B,E) was detectable in the cerebellum (black dashed circle) in sagittal (Fig. 3A,B) and transverse sections (Fig. 3D,E) during stages of onset of migration at 30 hpf. Furthermore, using the mAb735 antibody (Marx et al., 2001), PSA immunoreactivity was confined to *ncam*- and *stx*-expression domains, along the migratory route of cerebellar rhombic lip derived neuronal progenitors (Fig. 3C, arrows, F). These findings suggest that STX mediates NCAM polysialylation during migration of URL-derived neuronal progenitors.

Of interest, sagittal (Fig. 3G–I) and transverse (Fig. 3J–L) sections revealed that *ncam* expression remained broad throughout the cerebellum at 48 hpf (Fig. 3G,J), while the expression of *stx* declined close to the URL (Fig. 3H, red dashed circle) and became restricted to the rostral cerebellum (Fig. 3K, blacked dashed circle). Similarly, PSA immunoreactivity was found at this stage to delineate the anteroventral migratory pathway of neuronal progenitor cells in the dorsorostral cerebellum (Fig. 3L, black dashed circle), while PSA levels close to the URL diminished (Fig. 3I, red dashed circle).

PSA Removal Impairs Migration of URL-Derived Cerebellar Neuronal Progenitors

Given that PSA-NCAM is capable of regulating neuronal migration in mouse (Cremer et al., 1994; Ono et al., 1994) together with our findings that NCAM, STX, and PSA are coexpressed along the major neuronal migratory pathway in the differentiating cerebellum, we set out to investigate whether PSA-NCAM is capable of regulating URL-derived migration in the zebrafish cerebellum.

PSA is broadly expressed throughout the zebrafish nervous system already at 17 hpf and, thus, significantly earlier than the onset of URL-derived neuronal migration. We thus decided against a morpholino-mediated knockdown of polysialyltransferase expression as it lacks temporal control and does not allow discriminating between direct and indirect effects of PSA removal on neuronal migration in the developing cerebellum. In addition, injections of MO-STX1 (Marx et al., 2007) resulted only at very high concentrations (16 ng, approximately 2 nl of undiluted 1 mM stock) in a reduction but not complete absence of PSA together with malformations of embryos occurring before neuronal migration in the cerebellum. This finding suggests a weak functionality of the respective morpholino and requires careful controls in future functional studies to demonstrate the efficiency of anti–*stx*-morpholinos.

Therefore, recombinant EndoN enzyme, which selectively degrades polySia residues from NCAM (Rutishauser et al., 1985; Marx et al., 2001; Franz et al., 2005), was used to specifically remove PSA from the differentiating brain at 33 hpf, shortly after the onset of PSA-NCAM expression in the cerebellum (Marx et al., 2001). The efficiency of PSA removal in EndoN- and phosphate buffered saline (PBS) -treated control embryos was monitored by immunohistochemistry using the mAb735 anti-PSA antibody at 2 and 24 hr after injection. While control embryos displayed no obvious reduction in the levels of PSA at both time points (Fig. 4A,B, note that endogenous PSA levels decline at approximately 60 hpf), PSA was completely absent in the CNS of EndoN-injected embryos (Fig. 4C,D). This finding demonstrates that EndoN is capable to sufficiently degrade PSA

Fig. 3. Comparison of *ncam*, *stx* and polysialic acid (PSA) expression in the differentiating cerebellum during stages of neuronal migration. **A–L:** High magnification of lateral (A–C,G–I) and transverse (D–F,J–L) sections of the anterior hindbrain are shown at 30 hpf (A–F) and 48 hpf (G–L). The anterior cerebellar border at the midbrain–hindbrain boundary (MHB) and the posterior edge of the cerebellum are marked with a dashed and solid line, respectively. The mRNA in situ analysis was performed for *ncam* (A,D,G,J) and *stx* expression (B,E,H,K), while PSA immunoreactivity was detected using the mAb735 antibody (C,F,I,L). At 30 hours postfertilization (hpf), *ncam* expression was found to be strong and uniform in the cerebellum (A,D) similar to *stx* expression showing strongest expression at the ventricle (B,E). Moderate PSA levels can also be detected in this domain (C,F) during onset of rhombic lip derived neuronal migration (compare areas of dashed oval circles demarcating one cerebellar half). G,J: At 48 hpf, *ncam* expression is still uniform in the cerebellum. H,I: In contrast, *stx* and PSA expression have become restricted to the rostral cerebellar region along the MHB and are not longer detectable along the ventricle (red dashed circle). K,L: Transverse views of *stx* and PSA expression in the anterior cerebellum delineating the anteroventral migration route of neuronal progenitors. Scale bars = 50 μm.

Fig. 4. Polysialic acid (PSA) degradation in the central nervous system of EndoN-treated embryos impairs migration from the cerebellar rhombic lip. All panels show lateral views, anterior is to the left. Panels A–D show PSA expression in cerebellar primordia after phosphate buffered saline (PBS) and EndoN treatment at 33 hours postfertilization (hpf). **A,B:** Control embryos injected with PBS show PSA immunoreactivity after 2 hr (A) and 24 hr (B). Note the weaker expression levels at 57 hpf are due to endogenous down-regulation of PSA. **C,D:** EndoN injection at 33 hpf completely abolished PSA expression already after 2 hr (C), lasting up to 24 hr (D). Panels E–J are maximum intensity projections (50-μm stacks recorded every 12 min) showing migrating neuronal progenitors in the differentiating cerebellum after PBS (E–G) and EndoN treatment (H–J), recorded by in vivo time-lapse confocal microscopy. **E:** A group of neuronal progenitor cells at the upper rhombic lip (URL) in the control embryo is marked by a white arrowhead 2 hr post-injection. **F:** These cells migrated approximately halfway between URL and midbrain–hindbrain boundary (MHB) after 6 hr, while others have reached the MHB (white arrowhead). **G:** After 11 hr, cells in the ventral cerebellar region become stationary and form a cluster (white arrowhead, see Supplementary Movie S1). **H:** After injection of EndoN into the hindbrain ventricle, neuronal progenitors marked by an arrowhead are positioned at the URL or are polarized and span across the cerebellum between the URL and MHB (white arrowheads). **I,J:** Eight hours later (I), these neuronal progenitor cells are stalled at the URL and remain in this position (J) for up to 17 hr of time-lapse recording (see Supplementary Movie S2). K,L: The migratory routes of five individual uncGFP-expressing neuronal progenitor cells in PBS (K) and EndoN (L) -treated embryos were traced using the manual tracking tool plug-in of ImageJ1.34, the migratory direction is marked by an arrowhead. **K:** Anteroventral migration of cells in a PBS-injected embryo. **L:** Reduced migration of cells in EndoN-injected embryo. **M:** Quantification of neuronal progenitor migration from the cerebellar rhombic lip in PBS- and EndoN-treated embryos. While 84% of neuronal progenitors (n = 5 movies, 37 cells analyzed) in PBS-treated embryos were migratory, covering an average distance of almost 30μm during a 4-hr period, only 23% of neuronal progenitors (n = 5 movies, 47 cells analyzed) in EndoN-treated embryos were migratory and covered a similar average migration distance. Cells were classified as nonmigratory when they moved less than one cell diameter (approximately 10μm) during the observation period.

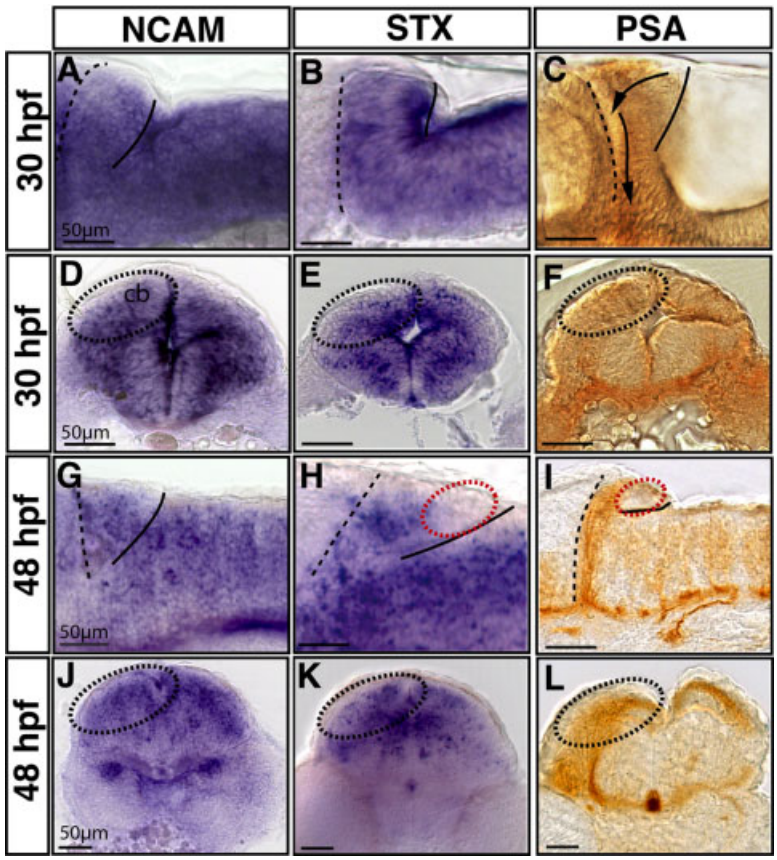

Fig. 3.

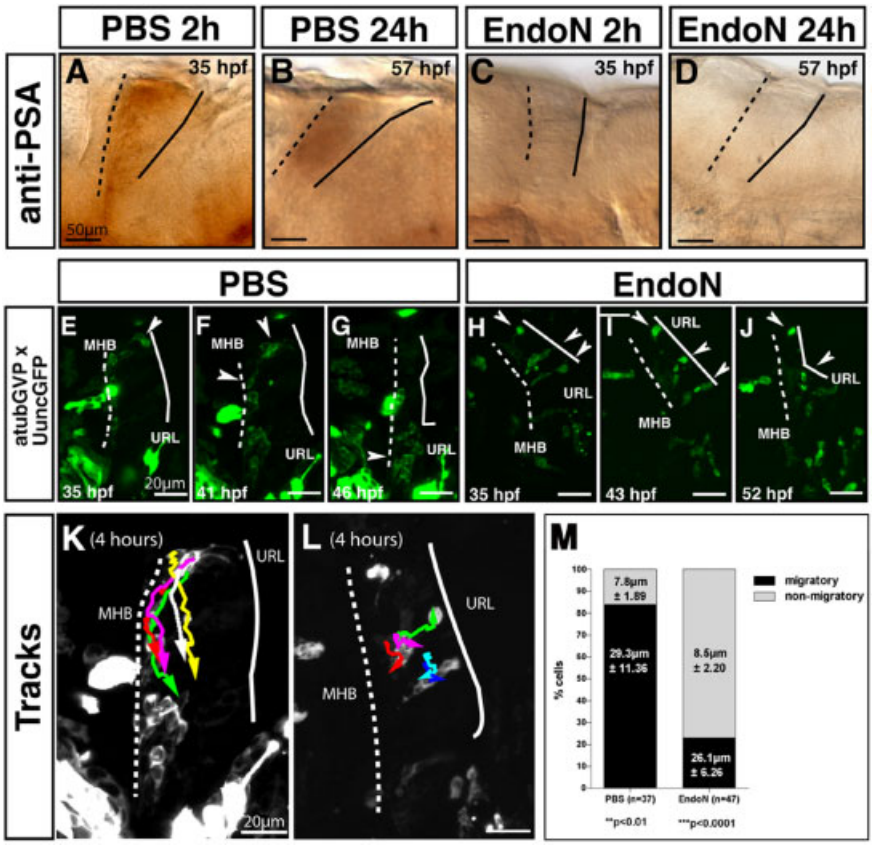

Fig. 4.

from neuronal progenitors in the differentiating cerebellum for at least a 24-hr period.

Altered levels of PSA in the differentiating CNS after EndoN injection were not expected to result in any gross morphological changes. Thus, we used time-lapse confocal microscopy to investigate cerebellar migration on a single-cell level. Neuronal progenitors were labeled in a mosaic manner through co-injection of atubGVP and UuncGFP expression vectors (25 pg each) into one-cell stage embryos as previously described (Köster and Fraser, 2001a,b). At 33 hpf, either 3–4 nl of PBS or 100 ng/µl EndoN/PBS was injected into the hindbrain ventricle of these green fluorescent protein (GFP) -expressing embryos and time-lapse imaging was performed starting 2 hr after injection (see Supplementary Movies S1 and S2, which can be viewed at http://www.interscience.wiley.com/jpages/1058-8388/suppmat).

In control embryos, clusters of labeled neuronal progenitor cells initiated migration by becoming polarized and extending leading processes into the direction of the MHB (Fig. 4E, white arrowhead). These progenitors then followed the characterized anteroventral pathway toward and along the MHB (Fig. 4F) to ventral regions of the anterior hindbrain (Fig. 4G, see also Supplementary Movie S1, n = 5 movies, 37 cells analyzed). Additional tracing of individual cells over a time period of 4 hr using the manual tracking tool plug-in of the ImageJ software (plug-in: Farbrice Cordelieres, Inst. Curie, Orsay, France; Abramoff et al., 2004) further demonstrated the proper anteroventral migration pathway of these cells from the URL toward and along the MHB (Fig. 4K, arrows point in the direction of migration). In contrast, URL-derived neuronal progenitors in EndoN-injected embryos exhibited dramatic migration defects after PSA degradation (see Supplementary Movie S2, n = 5 movies, 47 cells analyzed). Already 2 hr after EndoN-injection, URL-positioned neuronal progenitors failed to form a stable leading process, despite filopodial activity (Fig. 4H, white arrowheads). Consequently, such progenitors, although being polarized at the beginning, never initiated migration and ceased leading

process formation after 8 hr (Fig. 4I, white arrowheads). These cells never pursued anteroventral migration and remained stalled at a position close to the URL during the entire period of time-lapse recordings (Fig. 4 I,J white arrowheads). This reduced or complete lack of migration became further evident by individual cell tracing during a 4-hr period (Fig. 4L), showing that neuronal progenitors in EndoN-injected embryos remained in close proximity to their original position.

Additional quantification revealed that, in PBS-injected embryos, approximately 84% of neuronal progenitors (31/37) migrated significant distances during a 4-hr period covering an average distance of 29.3μm (SD ± 11.4μm), while 16% (6/37) of the cells remained at the URL or in close proximity, moving an average of 7.8μm (SD ± 1.9μm) and, thus, less than a cell diameter (Fig. 4M left column; $P < 0.01$). In contrast, only 23% of URL-derived neuronal progenitors (11/47) in EndoN-injected embryos were clearly migratory (average migratory distance: 26.1 μm SD ± 6.3 μm), while the majority of these neuronal progenitors (77%, 36/47) were nonmigratory (average migratory distance: 8.5 μm SD ± 2.2 μm) and remained stalled close to the URL (Fig. 4M right column; $P < 0.001$). Thus, our in vivo time-lapse analyses and quantifications of cell migration reveal that PSA is essential for regulating cellular motility of migratory neurons emanating from the zebrafish cerebellar rhombic lip.

DISCUSSION

In this report, we have addressed the spatiotemporal polysialylation of the adhesion factor NCAM in zebrafish by means of cloning zebrafish homologues of the highly conserved polysialyltransferases STX and PST and characterizing their expression patterns. We expand recent findings by characterizing their expression during stages of CNS differentiation, when migration and terminal differentiation of many neuronal populations occur.

Consistent with previous findings, STX remains the predominant polysialyltransferase expressed during brain differentiation (Marx et al., 2007). Interestingly, in addition to ventral diencephalic and tegmental brain regions, *stx* expression is mostly confined to dorsal hindbrain structures including the rhombic lip, from where neuronal progenitors have been shown to initiate long-distance migration. In particular, in the cerebellum, neuronal migration initiated from the cerebellar rhombic lip occurs toward and along the MHB (Köster and Fraser, 2006). The *stx* expression together with expression of *ncam* and PSA was found to delineate this migratory pathway.

In contrast to a very remote or lack of *pst* expression reported for embryonic stages (Marx et al., 2007), we find expression of *pst* in the differentiating zebrafish brain in ventral regions, but generally weaker when compared with *stx*. The *pst* expression is found in the hindbrain reaching from the dorsally positioned lower rhombic lip, which also expresses high levels of *stx*, toward the floorplate along the central domains of each rhombomere, where neuronal differentiation occurs (Cheng et al., 2004). At 4 days of embryonic development, expression of both polysialyltransferases has declined to minute amounts. During these embryonic stages, the peak of neuronal migration in the hindbrain and particular in the cerebellum has passed and terminal differentiation of neuronal populations is well under way (K. Volkmann et al., in press). These findings show that in addition to axonal pathfinding (Marx et al., 2001), polysialylation of NCAM could well play a role in regulating neuronal migration in zebrafish, such as in higher vertebrates (Murakami et al., 2000).

Based on the partially overlapping expression of *stx* and *pst* in the zebrafish brain, both enzymes could well play a synergistic or redundant function in modulating NCAM adhesion. Thus, rather than interfering with polysialyltransferase activity, the removal of PSA is a more robust assay to address a potential PSA-NCAM function during zebrafish neuronal migration. We have imaged rhombic lip-derived migration in the differentiating cerebellum, revealing that lack of PSA results in impaired cell migration. The observation that neuronal progenitors remained stalled in the vicinity of the URL after PSA removal suggests that polysialylated NCAM is required for mediating cellular motility. These findings are in good agreement with results from migrating neuronal progenitors in the rostral migratory stream in mammals. Here, tangential migration in mice lacking PSA-NCAM is severely decreased mainly in a cell-autonomous manner, leading to a significant size reduction of the olfactory bulb (Cremer et al., 1994; Chazal et al., 2000). However, a role for PSA in regulating early migration in the developing mouse cerebellum has remained elusive so far.

Recently, the cerebellar rhombic lip in mice has been shown to produce different neuronal cell populations over time (Wang et al., 2005; Machold and Fishell, 2005). Similarly, in the zebrafish, cerebellum rhombic lip derived migration starts at approximately 28 hpf, but granule progenitor cells do not initiate migration from the URL before 50 hpf, reaching its peak of migration between 60 and 80 hpf (K. Volkmann et al., in press). Thus, high expression levels of *ncam*, *stx*, and PSA in the zebrafish cerebellum, and successively declining levels of *stx* and PSA after 48 hpf, are consistent with PSA-NCAM specifically regulating early phases of rhombic lip derived migration. Intriguingly, the later arising granule progenitor cells rely on a cadherin-mediated adhesion system for proper migration (S. Rieger et al., unpublished results). This finding suggests that different adhesion systems and their specific spatiotemporal regulation may discriminate between different neuronal populations within the cerebellum to regulate their migratory behavior.

Our expression analysis in the adult zebrafish brain contrasts previous results, in which *pst* expression was reported to be absent (Marx et al., 2007). Similarly to the embryonic situation, we observed many cooperative expression domains of PST and STX throughout the mature brain such as in the RMS and olfactory bulb, the cerebellum or in the hypothalamus. Within these tissues the polysialyltransferases show both unique cell-type specific expression, such as in the caudal lobe of the cerebellum (*stx* only), and coexpression, such as in the hypothalamic lateral ventricles (*pst* and *stx*). These findings suggest that

PST and STX activity could act in an individual or synergistic manner in the adult CNS. Their expression is confined to many regions of adult neurogenesis and neuronal migration, which occurs more widespread in zebrafish than in higher vertebrates (Zupanc et al., 2005; Adolf et al., 2006; Grandel et al., 2006). For example, *pst* and *stx* expression is found in the subpallial stripe, the zebrafish equivalent of the mammalian RMS (Adolf et al., 2006) and in other ventricular regions such as in the periventricular gray zone of the optic tectum or the hypothalamus. Notably, in the cerebellum, complementary *stx* and *pst* expression is found in adjacent neuronal layers of synaptic partners. In the Purkinje cell layer, high expression levels of *stx* and low levels of *pst* could be detected, whereas low *stx* and high *pst* expression levels were found in the granule cell layer. Of interest, PSA is restricted to the axons (parallel fibers) and dendrites of the cerebellar granule cells but excluded from the soma (not shown). Therefore, based on the differential polysialyltransferase expression pattern in the mature cerebellum variances in polysialylation could play a role in mediating synaptic plasticity as it has been shown in mice, where PSA and polysialyltransferases can influence synaptic plasticity and long-term potentiation (Becker et al., 1996; Muller et al., 1996; Eckhardt et al., 2000). Furthermore, mice lacking polysialylated NCAM display deficits in spatial learning (Cremer et al., 1994; Becker et al., 1996).

Thus, as in higher vertebrates and mammals, spatiotemporal NCAM polysialylation is highly regulated during development and adulthood. While we have shown that PSA-NCAM is required for proper cell migration in the differentiating cerebellum, the dynamic expression of the polysialyltransferases suggests further functions during neurogenesis, axon pathfinding (Marx et al., 2001), synaptogenesis, and synaptic plasticity in memory and learning. This finding shows that polysialyltransferase activity in zebrafish may be more conserved to mammals than has been previously thought (Marx et al., 2007). Many of these functions when disrupted are not likely to result in gross morphological changes and will require more sophisticated phenotype analysis such as time-lapse imaging, electrophysiology, or behavioral studies. Given the accessibility of zebrafish for genetics, in vivo imaging and behavioral tests, analyzing PSA-NCAM function in zebrafish will be a rewarding research area. In particular, when addressed in the context of the high regenerative capacity of the zebrafish adult CNS, the regeneration-promoting activities of polysialyltransferases may reveal valuable insights for neuroregenerative approaches (Lavdas et al., 2006; Zhang et al., 2007).

EXPERIMENTAL PROCEDURES

Fish Strains and Maintenance

Raising, spawning, and maintaining of zebrafish lines were performed as described previously (Kimmel et al., 1995; Westerfield, 1995). For embryonic in situ hybridization, immunohistochemical analyses, and confocal time-lapse microcopy fish of the brass strain were used. The transgenic line islet1:GFP has been described (Higashijima et al., 2000). For adult paraffin sections, 9-month-old fish of the wild-type AB strain were used.

Cloning of Polysialyltransferases *pst* and *stx*

The zebrafish homologues of the polysialyltransferases STX and PST were isolated by RT-PCR using cDNA from 54 hpf embryos. The following primers were used: PSTup: TCTCGAGATGCGGCTTTCACG; PSTlow: TAGATTAAGTTGATGTGCATTTAGATGTC; STXup: TACTCGAGATGTCTTTTGAATTCCGAATACTGA; and STXlow: TATCTAGATCATGTAGGAGGTTTGCATGGTCCC. Amplified fragments of expected size (PST: ~1,070 bp; STX: ~1,150 bp) were subcloned into pCRII-vector (Invitrogen, San Diego, CA). Sequences were identical to those recently published NM 153662 (STX), NM 001099416 (PST; Harduin-Lepers et al., 2005; Marx et al., 2007).

RNA Expression Analysis

Both zebrafish polysialyltransferases and NCAM expression was analyzed by in situ hybridization on embryonic whole-mounts and adult paraffin sections. Zebrafish NCAM cDNA was obtained from (Mizuno et al., 2001). Digoxigenin-labeled antisense RNA (Roche, Germany) of *stx, pst*, and NCAM were hybridized on whole-mounts at 57°C and on paraffin sections at 68°C. For sagittal and transverse sections, whole-mount stained embryos were embedded in 5% agarose/1× PBS and 30- to 50-μm sections were cut on a Vibratome HM650V (MICROM, Germany). Paraffin-embedded adult brains were sectioned in 8-μm steps on a microtome 355S (MICROM, Germany). During incubations, sections were kept in a humidity chamber. Tween20 was omitted from all buffers except for the hybridization buffer. All stainings were photographed using an Axioplan2 microscope and Axiocam HRc camera (Zeiss, Germany) and processed with Adobe Photoshop7.0.

Immunohistochemistry

For PSA immunohistochemistry, embryos were acetone re-hydrated and blocked in 10% normal goat serum (NGS)/PBS-Tween20. DAB staining was performed after mAb735 anti-PSA (1:750) (Marx et al., 2001) and anti-mouse IgG-HRP antibody incubation (1:300; Jackson ImmunoResearch, West Grove, PA). Immunohistochemistry on islet1:GFP transgenic embryos at 48 hpf was performed after in situ hybridization for *stx*. Embryos were blocked in 10% NGS/PBS-Tween20 for 1 hr and incubated in a 1:500 dilution of rabbit anti-GFP antibody (Torrey Pines Biolabs, Houston, TX) overnight at 4°C. Secondary antibody incubation was performed using a 1:1,000 dilution of Cy2-conjugated anti-rabbit IgG antibody (Dianova, Germany).

Microinjection

For vital labeling of migrating neuronal progenitors, 25 pg each of the vectors atub-GVP and UuncGFP (Köster and Fraser, 2001b) in 0.02% Phenol Red/H_2O were co-injected into one-cell stage embryos. Embryos at 33 hpf ex-

pressing GFP in cells of the cerebellar rhombic lip were further injected into the fourth ventricle with 3–4 nl of 1× PBS or EndoN/PBS at 100 ng/μl. Time-lapse recordings were started 2 hr after injection.

Intravital Imaging

Mounting and image recording for three-dimensional time-lapse confocal microscopy was performed as described (Köster and Fraser, 2004) using a LSM510 Meta laser-scanning microscope (Zeiss, Germany). Image data were analyzed and processed with the Zeiss LSM-Software version 4.5 and Adobe Photoshop7.0.

Quantification of Migration

Tracing of UuncGFP-labeled cerebellar neuronal progenitors was performed over a 4-hr period using the manual tracking tool plug-in of the open source software ImageJ1.34s (plug-in: Farbrice Cordelieres, Inst. Curie, Orsay, France; Abramoff et al., 2004). Statistical analyses were performed using the GraphPad software Prism4.

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