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Ncam1a and Ncam1b: Two carriers of polysialic acid with different functions in the developing zebrafish nervous system

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Polysialic acid (polySia) is mainly described as a glycan modification of the neural cell adhesion molecule NCAM1. PolySia-NCAM1 has multiple functions during the development of vertebrate nervous systems including axon extension and fasciculation. Phylogenetic analyses reveal the presence of two related gene clusters, *NCAM1* and *NCAM2*, in tetrapods and fishes. Within the *ncam1* cluster, teleost fishes express *ncam1a (ncam)* and *ncam1b (pcam)* as duplicated paralogs which arose from a second round of ray-finned fish-specific genome duplication. Tetrapods, in contrast, express a single *NCAM1* gene. Using the zebrafish model, we identify Ncam1b as a novel major carrier of polySia in the nervous system. PolySia-Ncam1a is expressed predominantly in rostral regions of the developing nervous system, whereas polySia-Ncam1b prevails caudally. We show that *ncam1a* and *ncam1b* have different expression domains which only partially overlap. Furthermore, Ncam1a and Ncam1b and their polySia modifications serve different functions in axon guidance. Formation of the posterior commissure at the forebrain/midbrain junction requires polySia-Ncam1a on the axons for proper fasciculation, whereas Ncam1b, expressed by midbrain cell bodies, serves as an instructive guidance cue for the dorso-medially directed growth of axons. Spinal motor axons, on the other hand, depend on axonally expressed Ncam1b for correct growth toward their target region. Collectively, these findings suggest that the genome duplication in the teleost lineage has provided the basis for a functional diversification of polySia carriers in the nervous system.

Keywords: development / NCAM / nervous system / polysialic acid / zebrafish

Introduction

Polysialic acid (polySia), a large homopolymer of α-2,8-linked sialic acid, is a unique glycan polymer found on only a small subset of vertebrate proteins. The neural cell adhesion molecule NCAM1 has so far been regarded as the dominant carrier of polySia (Edelman and Chuong 1982; Finne 1982; Hoffman et al. 1982; Finne et al. 1993). NCAM1 is the best characterized member of the immunoglobulin (Ig) superfamily of cell adhesion molecules which are involved in many aspects of the development of vertebrate nervous systems. NCAM1 mediates (i) homophilic interactions between neighboring cells, (ii) heterophilic interactions between cells and components of the extracellular matrix and (iii) homo- or heterophilic interactions with components in *cis* (for a recent review, see Ditlevsen et al. 2008). Thereby, NCAM1 plays important roles in cell adhesion (Covault and Sanes 1985; Kasper et al. 2000; Walmod et al. 2004), cell proliferation and migration, neuritogenesis, axon guidance and fasciculation (Rutishauser and Landmesser 1996; Prag et al. 2002; Seidenfaden et al. 2006; Burgess et al. 2008), synaptic plasticity and regeneration (Gascon et al. 2007; Rutishauser 2008).

PolySia is attached to the fifth Ig-like domain of NCAM1 by two polysialyltransferases [St8Sia2 (St8SiaII, STX) and St8Sia4 (St8SiaIV, PST); Angata and Fukuda 2003]. It is highly up-regulated in the nervous system during the embryonic development of mammals and fishes and drastically down-regulated post-natally or around hatching, respectively (Rutishauser 2008; Gascon et al. 2010; Hildebrandt et al. 2010; Kleene and Schachner 2010). Due to its large volume, polySia inhibits NCAM1 homophilic binding; it thereby increases intermembrane repulsion and abrogates cell adhesion (Johnson et al. 2005). In addition, it affects other NCAM1-dependent cell surface interactions in several ways (reviewed by Hildebrandt et al. 2007). Consequently, functions of NCAM1-attached polySia have been implicated in cell migration, axon outgrowth and fasciculation, neural

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plasticity, hippocampal long-term potentiation and repair (Bonfanti 2006; Hildebrandt et al. 2007; Burgess et al. 2008; Rutishauser 2008; Bonfanti and Theodosis 2009; Mühlenhoff et al. 2009; El Maarouf and Rutishauser 2010; Kleene and Schachner 2010; Li et al. 2011).

We have previously extended the analysis of polySia functions to the zebrafish model system and we uncovered its effects during the formation of several axonal tracts. Axons of the posterior commissure (pc) carry polySia and cross the forebrain/midbrain junction in a region of polySia-expressing cells. Surprisingly, the removal of polySia from this structure causes the pc to split into several bundles (Marx et al. 2001). This suggests that polySia balances adhesive interactions among the axons as well as between axons and their surroundings and that the removal of polySia interferes with this balance. In the hindbrain, polySia-free axons of commissural interneurons depend on polySia on the floorplate which presents a molecular cue for midline crossing (Marx et al. 2001). In addition to early functions, polySia presented by glia cells was recently implicated to aid the generation of rod photoreceptors from later developmental stages on until adulthood (Kustermann et al. 2010).

In addition to *NCAM1*, vertebrates express a second paralog, *NCAM2 (OCAM)*, whose function remains largely elusive (Paoloni-Giacobino et al. 1997; von Campenhausen et al. 1997; Yoshihara et al. 1997; Kulahin and Walmod 2010). Teleosts express yet a third member of the *ncam* family, *ncam1b* (*pcam*), as has first been demonstrated in zebrafish (Mizuno et al. 2001). (In this manuscript, we follow the current guidelines for naming nucleic acids and proteins as they were established by the relevant model organism nomenclature committees. We regret that the text reads balky in chapters discussing phylogenetic aspects.) Zebrafish Ncam1a, Ncam1b and Ncam2 share the stereotypic domain structure of five Ig-like domains and two fibronectin type III-like domains on the extracellular side, a transmembrane domain and a C-terminal cytoplasmic region, which is the region of highest dissimilarity among the three molecules (Mizuno et al. 2001). All three contain putative glycosylation motifs for polysialylation in the fifth Ig-like domain. By *in situ* hybridization, *ncam1a* and *ncam1b* were detected in overlapping regions of the zebrafish nervous system from early developmental stages on. The expression of *ncam2* is restricted to small clusters of secondary forebrain neurons in later developmental stages (Mizuno et al. 2001).

The evolutionary and functional consequences of a third member of the *ncam* gene family in teleosts are the main focus of the present study. *ncam1a* and *ncam1b* are characterized as duplicated paralogs in teleosts showing the same degree of similarity to the single tetrapod *NCAM* gene. By using specific antibodies, we show that Ncam1a and Ncam1b are widely expressed in the nervous system; they have distinct expression domains, which only partially overlap. We identify Ncam1b as a novel major carrier of polySia in the nervous system of teleost fishes, and we show that several parts of the nervous system differ significantly regarding their major polySia-modified Ncam paralog. Our results suggest that an elaborate interplay between polySia-Ncam1a and polySia-Ncam1b directs the guidance and fasciculation of different axon populations in the developing brain.

Results

Ncam1a and Ncam1b genes are duplicated paralogs in the teleost fish lineage

To understand the evolution of the *ncam* gene family in zebrafish, we studied the variation of these genes in teleost fishes and in representative tetrapods using phylogenetic analyses. All completely sequenced fish genomes contain at least three genes of the *ncam* family, whereas in tetrapods only two genes are detected. The phylogenetic relationship of the *NCAM* gene family reveals the presence of two related gene clusters (*NCAM1* and *NCAM2*, Figure 1) in both tetrapods and fishes. These groups appear to be monophyletic sister groups, suggesting that they arose as a result of an ancient duplication event which took place in a common ancestor of fishes and tetrapods. The internal clustering of genes within these groups is consistent with the known taxonomy. Our analyses identify the fish *ncam1a* and *ncam1b* genes as duplicated paralogs within the *ncam1* cluster. These paralogs are shared by teleost fishes in contrast to a single *NCAM1* gene found in all tetrapod species analyzed (Figure 1). The obtained phylogeny, however, does not allow answering whether one of the two *ncam1* paralogs of teleosts has retained the ancestral function and the other underwent neofunctionalization. The molecular distance-based topologies might suggest that the branches leading to clusters comprising both fish *ncam1* paralogs are unequally long and that fish *ncam1a* is more similar to the higher vertebrate *NCAM1* than fish *ncam1b.* The differences are, however, not large enough to allow further conclusions. We, therefore, assume that both paralogs are roughly equally distant from the ancestral state, suggesting that they were subjected to similar evolutionary forces. The same conclusions result from the study of synteny within the *ncam1* gene family, which reveals that genomic rearrangements have occurred equally frequent in the genomic surroundings of both fish *ncam1b* and *ncam1b* when compared with their tetrapod counterpart (not shown). Our results highlight the evolutionary conservation within the vertebrate *NCAM* gene family at the gene organization and protein structure levels as well as the duplication of the *ncam1* gene in teleost fishes.

In the zebrafish, ncam1a and ncam1b have distinct expression domains, which only partially overlap

An initial in situ hybridization study by Mizuno et al. (2001) had demonstrated that *ncam1a* and *ncam1b* are expressed in a partially overlapping pattern in the developing zebrafish brain. To test the evidences for a functional diversification of *ncam1* paralogs during the formation of the zebrafish nervous system, we re-investigated their expression patterns more precisely. Using specific antibodies in whole-mount immunostainings, we analyzed the distribution of Ncam1a, Ncam1b and Ncam2 on neuronal cell bodies and axons of embryos from 24 to 48 hpf (hours post-fertilization).

Expression of Ncam2 starts late during development (30 hpf), several hours after the formation of major axon tracts is completed and it is initially restricted to a few cells of the olfactory system (not shown). Ncam2 in the zebrafish embryo is not polysialylated (not shown), which is in agreement with the finding that, despite containing a putative glycosylation motif for polysialylation, its mouse ortholog (NCAM2,

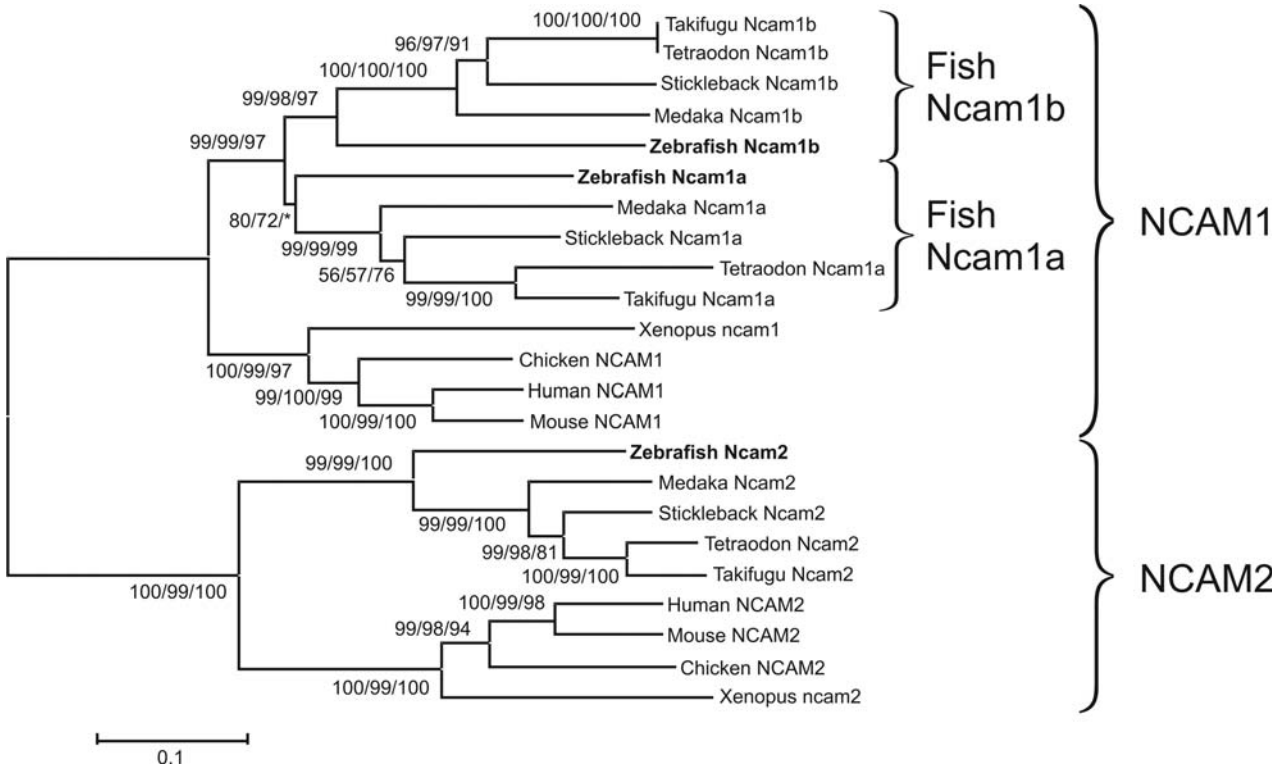

Fig. 1. Evolutionary relationships among members of the *Ncam* gene family. Unrooted NJ tree showing the evolutionary relationships among members of the NCAM gene family in teleosts and representative tetrapod species. Numbers at bifurcations indicate supporting bootstrap values (1000 replications) for the NJ, ME and MP analyses. An asterisk indicates the different topology suggested by the MP analysis. Branch lengths correspond to evolutionary distances indicated by the scale (in base substitutions per site).

OCAM) is not polysialylated as well (Yoshihara et al. 1997). Evidences are accumulating that the Ig5 domain of NCAM2 is not permissive for polysialylation (K. Colley, personal communication). We therefore focused on the expression of Ncam1a and Ncam1b. Both are clearly detected by antibody staining in the central nervous system (CNS) from 24 hpf onwards. Ncam1a shows a more or less ubiquitous expression on cell bodies of the all parts of the brain (Figure 2A), whereas Ncam1b is concentrated in the area surrounding the diencephalic/mesencephalic border, in the anterior midbrain and in the otic vesicle (Figure 2C). In the spinal cord, Ncam1a and Ncam1b are detected on cell bodies (Figure 2B and D). Both show a similar expression pattern on all cells with exception of the floorplate, which is exclusively stained by the Ncam1a antibody (Figure 2B).

Regions of overlapping expression and regions of exclusion become more evident at 36 hpf (Figure 3). Ncam1a is rather evenly distributed in the forebrain and midbrain, showing a strong expression in the olfactory placode and olfactory bulb, the eye, the pc, the cerebellum anlage and the hindbrain (Figure 3A). Ncam1b expression, on the other hand, is restricted to the olfactory bulb, the epiphysis and the area surrounding the pc (Figure 3B). In the cerebellum anlage and the hindbrain, Ncam1b staining is strong but less distinct than the Ncam1a staining (Figure 3A and B). Cells of the otic vesicle are characterized by robust Ncam1b expression (Figure 3F), whereas Ncam1a is restricted to a small population of cells in the rostral part of this structure (Figure 3E). The expression of both Ncam1a and Ncam1b in the CNS seems to be restricted to the cell bodies of neurons; the pc is the only axonal tract showing a distinct expression of Ncam1a (Figures 3A and 6). In the spinal cord as well, Ncam1a and Ncam1b are detected on cell bodies only. Whereas dorsal and medial parts express Ncam1a and Ncam1b rather evenly, the floorplate (Figure 3G and H), which is exclusively stained by the Ncam1a antibody (Figure 3G). In the peripheral nervous system, Ncam1a and Ncam1b are expressed on motor axons in the trunk and tail regions (Figure 3G and H) as well as on the cranial nerves nV, nVII (Figure 3C and D), nIX and nX and in the lateral line nerve (not shown).

In summary, both Ncam1a and Ncam1b are expressed in the zebrafish CNS. Whereas Ncam1a shows a stronger and more even expression pattern, Ncam1b is restricted to more specific brain regions.

Addition of polySia is a posttranslational modification of both Ncam1a and Ncam1b

As the expression patterns of Ncam1a and Ncam1b in the zebrafish (Figures 2 and 3) coincide with the localization of polySia (Marx et al. 2001), we determined whether one or both Ncam1 paralogs are polysialylated. Using morpholinos, we individually knocked down *ncam1a* or *ncam1b*, respectively, and we studied the polySia pattern in the resulting

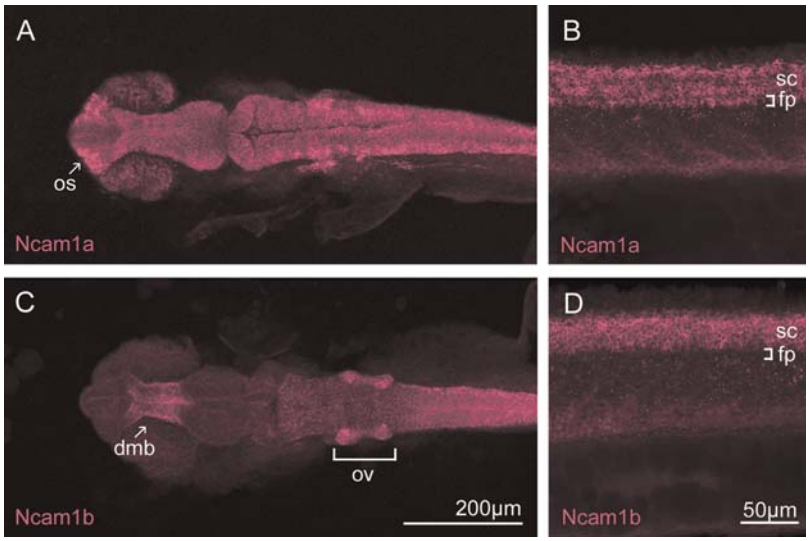

Fig. 2. Expression of Ncam1a and Ncam1b in the developing zebrafish nervous system at 24 hpf. Embryos at 24 hpf labeled with antibodies against Ncam1a (**A** and **B**) or Ncam1b (**C** and **D**). (A) and (C) dorsal view (rostral to the left). (B) and (D) lateral view (rostral to the left). (A) Ncam1a is uniformly expressed on cell bodies throughout the brain and in the spinal cord. Prominent expression is detected in the olfactory system (os). (B) Ncam1b is expressed in the area surrounding the diencephalic/mesencephalic border (dmb), the otic vesicle (ov) and the spinal cord (sc). Floorplate cells (fp) of the sc exclusively express Ncam1a (B), whereas the other cells of the sc express both Ncam1a and Ncam1b (B and D).

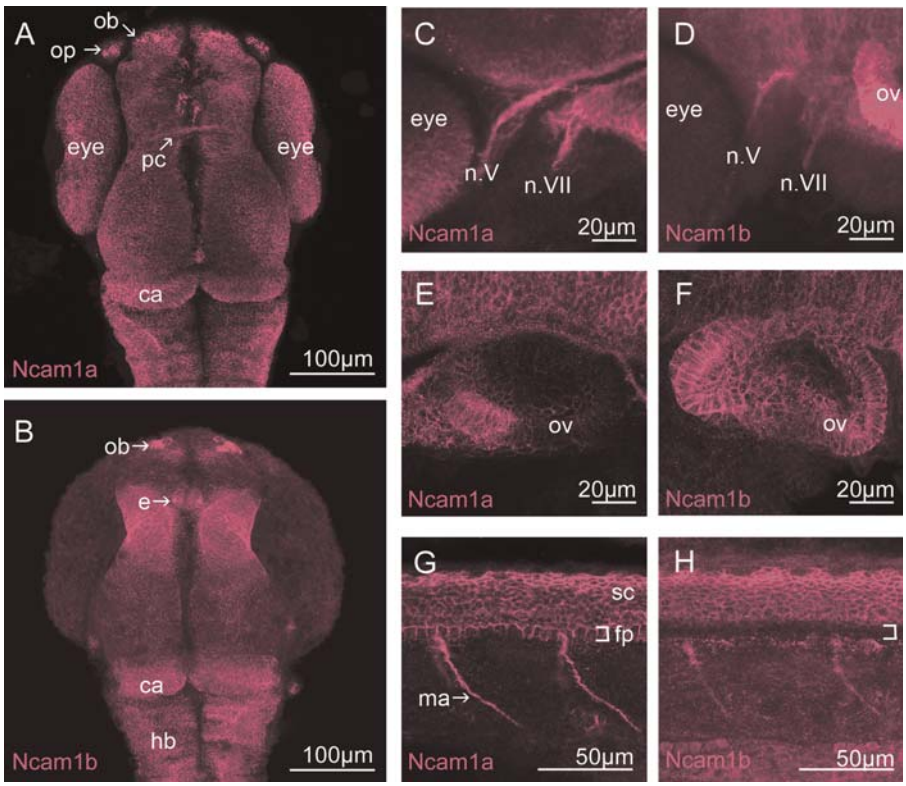

Fig. 3. Expression of Ncam1a and Ncam1b in the developing zebrafish nervous system at 36 hpf. Embryos at 36 hpf labeled with antibodies against Ncam1a (**A**, **C**, **E** and **G**) or Ncam1b (**B**, **D**, **F** and **H**). (A) and (B) dorsal view of the head. (C)–(H) lateral view (rostral to the left). (A) Ncam1a is uniformly expressed on cell bodies throughout the brain. Prominent expression domains include the olfactory bulb (ob), the olfactory placode (op), the pc, the eye and the cerebellum anlage (ca). (B) Ncam1b is expressed in the olfactory bulb (ob), the epiphysis (e), the dorsal midbrain, the cerebellum anlage (ca) and the hindbrain (hb). Motor axons of the peripheral nervous system here shown for cranial nerves V and VII, express Ncam1a (C) more abundantly than Ncam1b (D). The otic vesicle shows a robust expression of Ncam1b on all cell bodies (F), whereas expression of Ncam1a is restricted to the rostral part (E). (G and H) Cells of the floorplate (fp) of the spinal cord (sc) exclusively express Ncam1a (G), whereas the other cells of the sc and the motor axons (ma) express both Ncam1a and Ncam1b.

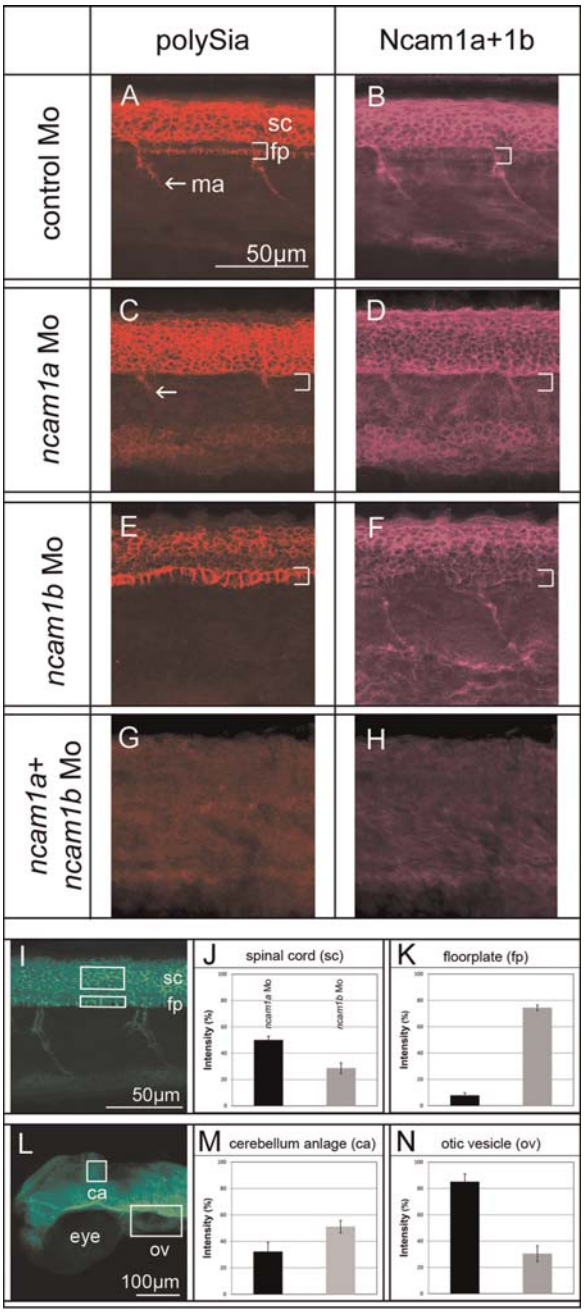

Fig. 4. Both Ncam1a and Ncam1b carry polySia. (**A**–**H**) Embryos at 36 hpf labeled with antibodies against polySia (mAb 735; A, C, E and G) and a polyclonal serum detecting both Ncam1a and Ncam1b (Ab 740; B, D, F and H). Lateral views of the zebrafish tail (rostral to the left). (A and B) Control morpholino-injected embryos express polySia on all cells of the spinal cord (sc), the floorplate (fp, bracket) and the motor axons (ma, arrow) (A). Labeling of Ncam1a and Ncam1b (B) correlates with the polySia staining (A). *ncam1a* knockdown leads to a complete loss of polySia on floorplate cells, whereas polySia is still detectable in the spinal cord and on motor axons (C). Ncam1a/Ncam1b immunoreactivity displays the same pattern (D). (E and F) *ncam1b* knockdown reduces polySia immunoreactivity in the spinal cord and on motor neurons, whereas floorplate cells remain unaffected (E). The reduction in Ncam1a/Ncam1b immunoreactivity in the spinal cord and the motor axons is less pronounced than the polySia reduction (F), suggesting that these structures express Ncam1a molecules without attached polySia. Double knockdown of *ncam1a* and *ncam1b* reduces polySia immunoreactivity (G) and Ncam1a/Ncam1b immunoreactivity (H) completely. Scale bar in (A) applies to (A)–(H). (**I**–**N**) Quantification of polySia expression: polySia was detected after morpholino knockdown of *ncam1a* or *ncam1b* in 36 hpf embryos by labeling with EndoN-GFP, a dose-dependent affinity probe and quantified by fluorescence microscopy. (I and L) control morpholino-injected embryos, lateral views (rostral to the left) of tail (I) and head (L) showing the regions selected for quantification. Boxes indicate spinal cord (sc) and floorplate (fp) in (I) and cerebellum anlage (ca) and otic vesicle (ov) in (L). (J–L) Fluorescence intensity of polySia labeling relative to control morpholino-injected specimen after morpholino knockdown of *ncam1a* (black bars) and *ncam1b* (gray bars) in spinal cord (*ncam1a*-MO: 50.1%, *ncam1b*-MO: 28.6%) (J); floorplate (*ncam1a*-MO: 7.8%, *ncam1b*-MO: 74.4%) (K); cerebellum anlage (*ncam1a*-MO: 32.3%, *ncam1b* -MO: 52.5%) (M); and otic vesicle (*ncam1a*-MO: 85.1%, *ncam1b* -MO: 30.5%) (N).

morphants. Both morpholinos effectively and specifically suppress the expression of the targeted gene, as is demonstrated by immunostaining with specific antibodies for Ncam1a or Ncam1b (Supplementary data, Figure S1). Specificity of the knockdown phenotype is further confirmed by mRNA rescue experiments (Figure 7H). Individual or double knockdowns of *ncam1a* or *ncam1b* do not cause a marked increase in mortality of the embryos (Supplementary data, Figure S2). Apparently, neither cell adhesion molecule is unconditionally required for zebrafish development. We did, however, experience an increased frailness of knockdown embryos during immunostaining and embedding, indicative of an overall decrease in tissue connectivity in these specimens.

Single knockdowns of either *ncam1a* or *ncam1b* induce specific modifications of the polySia pattern, as is revealed by immunostainings using a monoclonal antibody against polySia and a polyclonal antiserum detecting both Ncam1a and Ncam1b (Figure 4C and E). The latter antibody was used in this experiment to enable a direct comparison of the expression levels of both Ncam paralogs. In *ncam1a* knockdown embryos, polySia staining is completely abolished in the floorplate of the spinal cord, whereas other cells are only slightly affected (Figure 4C). Knockdown of *ncam1b*, in contrast, noticeably reduces polySia expression in the spinal cord but does not obviously affect polySia on the floorplate (Figure 4E). A total reduction in polySia is only achieved by double knockdown of *ncam1a* and *ncam1b* (Figure 4G). These results show that in zebrafish both Ncam1a and Ncam1b, carry the polySia modification. Motor axons express both Ncam1a and Ncam1b, as becomes evident when comparing single and double knockdowns (Figure 4D, F and H). Most, if not all, polySia on the motor axons seems to be attached to Ncam1b, since *ncam1b* knockdown reduces polySia immunoreactivity to below the detection level (Figure 4E).

To achieve a spatially resolved quantification of polySia carriers, we detected polySia with an affinity probe, endosialidaseN (EndoN)-green fluorescent protein (GFP), which serves as a dose-dependent single-step reagent for substrate labeling and quantification in fluorescence microscopy (Jokilammi et al. 2004). In the spinal cord, application of the *ncam1b* morpholino leads to a stronger reduction in polySia (gray bar in Figure 4J) than application of the *ncam1a* mor-

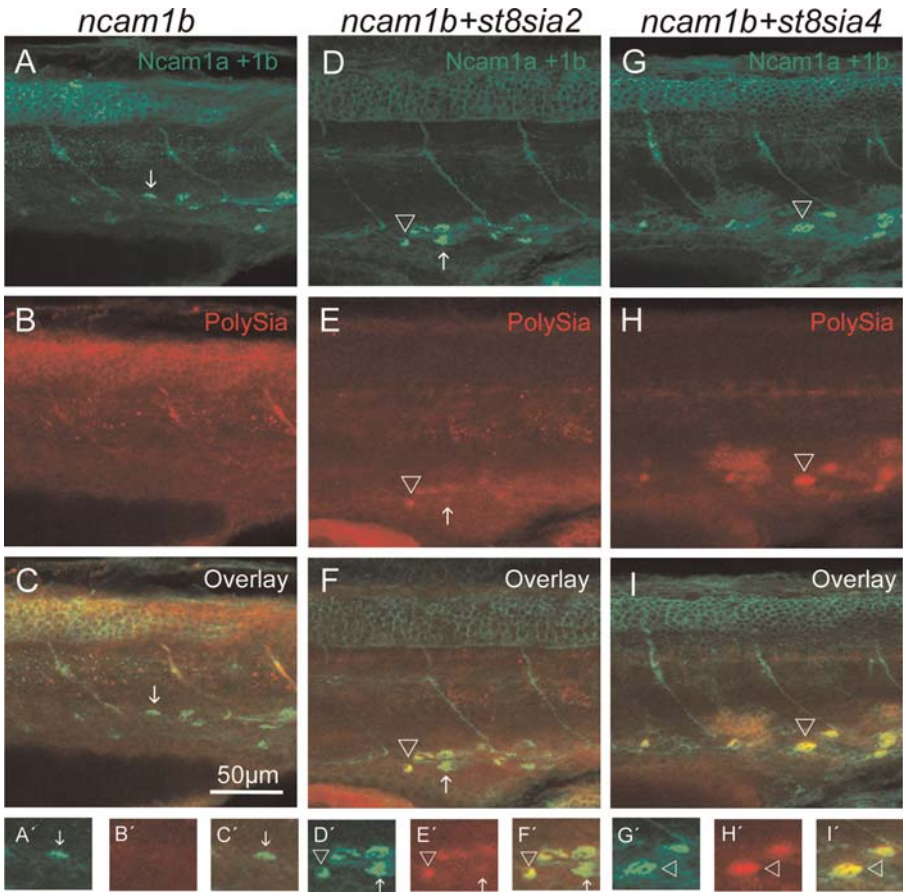

Fig. 5. Ncam1b is a substrate for polysialylation by both St8Sia2 and St8Sia4. Wild-type embryos were injected at the 1-cell stage with Tol2 plasmids encoding Ncam1b (**A**–**I** and **A′**–**I′**), St8Sia2 (D–F and D′–F′) or St8Sia4 (G–I and G′–I′) driven by the α-actin promoter. Embryos were fixed at 36 hpf and double immunostained with a polyclonal serum detecting both Ncam1b and Ncam1b (Ab 740; green) and antibodies against polySia (mAb 735; red) as indicated. Individual stainings or overlays are indicated in the figure. (A′)–(I′) show enlargements of (A)–(I). Injections induce a punctate expression of Ncam1b in the ventral portion of the myotomes (arrows and arrowheads). When Ncam1b is co-expressed with either St8Sia2 (D–F) or St8Sia4 (G–I), a fraction of these puncta contain polySia (arrowheads) indicative of polysialylated Ncam1b (E, F, H and I). Arrows indicate the patches of non-polysialylated Ncam1b.

pholino (black bar in Figure 4J), indicating that Ncam1b is the major carrier of polySia in this structure. Whereas about two of three of polySia on neurons of the spinal cord is attached to Ncam1b, floorplate cells contain more than 7-fold as much polySia-Ncam1a than polySia-Ncam1b (Figure 4K). PolySia expression on motor axons is too weak to enable a reliable quantification by EndoN-GFP labeling. Similar to the spinal cord, the rostral CNS consists of areas which are rather specialized with regard to the polySia carrier and of areas expressing roughly equal amounts of polySia-Ncam1a and polySia-Ncam1b. The latter case is exemplified by the cerebellum anlage. Here, the amount of EndoN-GFP is reduced to ∼30% after *ncam1a* knockdown (black bar in Figure 4M) compared with a reduction in ∼50% after *ncam1b* knockdown (gray bar in Figure 4M). A rather specialized region in the rostral brain is the otic vesicle which expresses significantly more Ncam1b than Ncam1a (Figure 3E and F). Accordingly, polySia quantification after morpholino knockdown reveals a 3:1 ratio of polySia-Ncam1b:polySia-Ncam1a in the otic vesicle (Figure 4N).

Taken together, we show that Ncam1b, besides Ncam1a, is a prominent carrier of polySia in the zebrafish nervous system. Whereas polySia-Ncam1a is expressed predominantly in rostral regions, polySia-Ncam1b prevails caudally. Exceptions of this rule are the otic vesicle and the midbrain region surrounding the pc, which carry mostly polySia-Ncam1b, and the floorplate of the spinal cord, which expresses exclusively polySia-Ncam1a.

Ncam1a and Ncam1b are substrates for polysialylation by both polysialyltransferases St8Sia2 and St8Sia4

Two polysialyltransferases synergistically interact in generating polySia on mammalian NCAM, namely St8Sia2 and St8Sia4 (Nakayama et al. 1998). In the zebrafish embryo, however, St8Sia2 appears to be the sole enzyme responsible for polysialylation, as morpholino knockdown of *st8sia2* results in a reduction in polySia to levels below the detection limit (Marx et al. 2007). The second polysialyltransferase encoded by the zebrafish genome, St8Sia4, is expressed in later developmental stages, rather weakly and only in small cell populations (Marx et al. 2001; Rieger et al. 2008; Chang et al. 2009). To study the ability of both polysialyltransferases to act on both Ncam paralogs, Ncam1a and Ncam1b, we ectopically expressed *ncam1a* or *ncam1b* in combination with

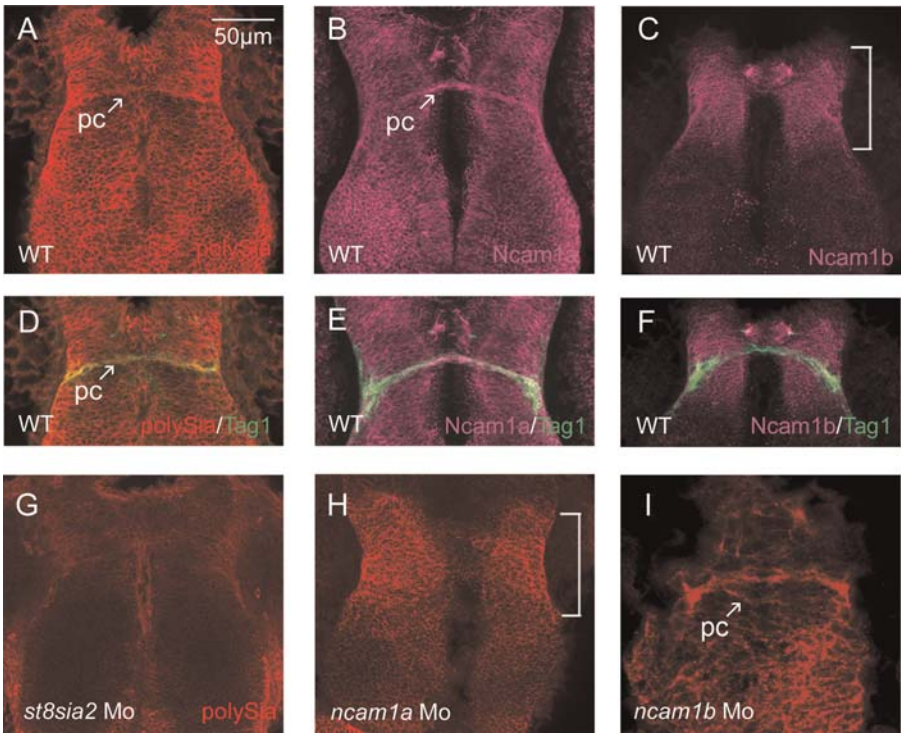

Fig. 6. Complementary expression patterns of Ncam1a, Ncam1b and polySia in the midbrain region surrounding the pc. (**A**–**I**) Confocal images showing the heads (dorsal views, rostral to the top) of zebrafish embryos at 36 hpf immunostained for polySia (red), Ncam1a (magenta), Ncam1b (magenta) or Tag-1 (green) to label axons of the pc, as indicated. Scale bar in (A) applies to (A)–(F) and in (G) to (G)–(I). (A–F) In wild-type embryos, polySia and Ncam1a are expressed on almost all cells in the midbrain and on axons of the pc (arrows). Ncam1b expression is restricted to cells surrounding commissural axons (bracket in C), and Ncam1b is not detected on the axons themselves. (G–I) PolySia staining of embryos after morpholino knockdown of polysialyltransferase *st8sia2*, *ncam1a* or *ncam1b*, respectively. (G) Knockdown of *st8sia2* leads to an almost complete loss of polySia immunoreactivity. (H) Knockdown of *ncam1a* removes polySia on pc axons and in posterior and anterior regions of the midbrain. (I) Knockdown of *ncam1b* leads to an almost complete reduction in polySia on cells surrounding the pc, but does not affect polySia on the axons themselves (arrow) and in anterior and posterior parts of the midbrain.

either *st8Sia2* or *st8sia4* in skeletal muscle cells of zebrafish embryos by the use of the Tol2 system (Kwan et al. 2007). No endogenous Ncam1a, Ncam1b, polysialyltransferases or polySia are detected in this tissue (Marx et al. 2001; Mizuno et al. 2001; Rieger et al. 2008; Chang et al. 2009; Figures 2 and 3). We find that the α-actin promoter used for these studies drives a punctate expression of Ncam1b in the ventral portion of the myotomes (Figure 5A, D and F), and we find polySia co-localized with a fraction of these puncta if St8Sia2 or St8Sia4 are co-expressed (Figure 5E and H). The same holds for Ncam1a (Supplementary data, Figure S3). Consequently, St8Sia2 and St8Sia4 are able to polysialylate both Ncam1a and Ncam1b.

PolySia-Ncam1a and polySia-Ncam1b have distinct roles in regulating the growth patterns of commissural and motor axons

The widespread, yet distinct, expression of Ncam1a and Ncam1b suggests discrete functions during the development of the CNS which are expected to be modulated by polysialylation. An established model to study the axonal guidance in the zebrafish is the pc, whose axons critically depend on the presence of polySia for correct projection and bundling. The pc is formed between 20 and 22 hpf by a few pioneering axons which extend from neurons located laterally in the rostral midbrain (Chitnis and Kuwada 1990; Ross et al. 1992; Hjorth and Key 2002; Marx et al. 2007). Roughly 100–200 axons are added to the pc between 30 and 38 hpf (Wilson et al. 1990). These axons cross dorsally at the junction of the forebrain and the midbrain in a single fasciculated bundle. We have previously shown that pc axons split into several tightly fasciculated bundles after the removal of polySia (Marx et al. 2001, 2007). Here, we reinvestigate this behavior with respect to Ncam1a and Ncam1b.

Immunostainings of control embryos (Figure 6A–F) and of embryos after morpholino knockdown of *ncam1a, ncam1b* or *st8sia2* (Figure 6G–I) demonstrate the complementary expression patterns of Ncam1a, Ncam1b and polySia in the midbrain region surrounding the pc which is summarized as follows (Figure 9): (i) axons in the pc express only polySia-Ncam1a, (ii) the pc grows in a region of the forebrain/midbrain junction with neuronal cell bodies expressing polySia-Ncam1b and Ncam1a without polySia modification and (iii) more rostral and more caudal regions express polySia-Ncam1a but no Ncam1b.

Consistent with the distinct expression patterns, morpholino knockdowns of either *ncam1a* or *ncam1b* affect the growth

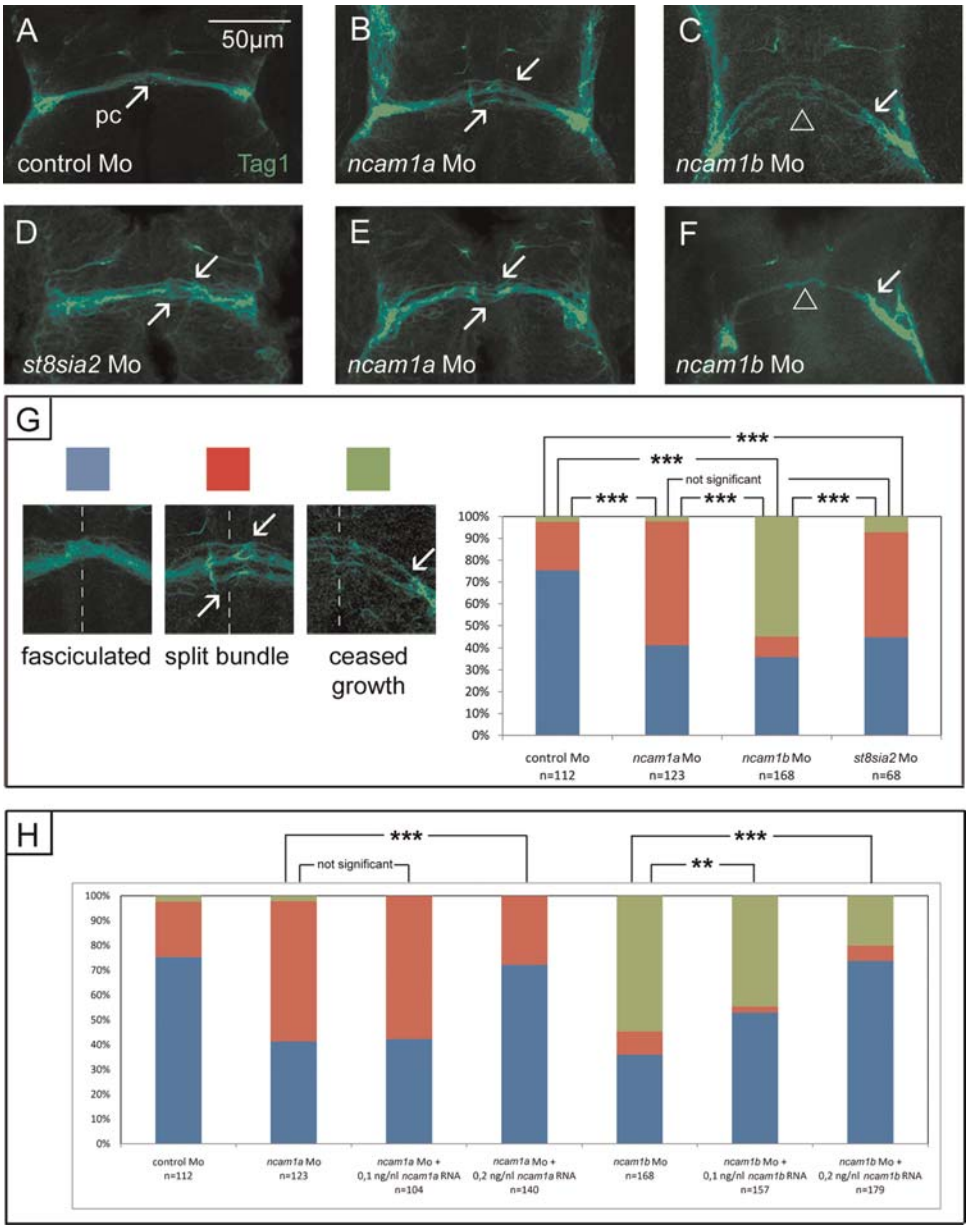

Fig. 7. Ncam1a, Ncam1b and polySia affect the axonal growth in the pc. (**A**–**F**) pc's (dorsal views, rostral to the top) of zebrafish embryos at 36 hpf immunostained for Tag-1 after morpholino injection. Scale bar in (A) applies to (A)–(F). (A) In control morpholino-injected embryos, pc axons grow in a fasciculated bundle (arrow). (D) In *st8sia2* knockdowns, the pc splits into several bundles (arrows). (B and E) In *ncam1a* knockdown embryos, the pc splits up into several bundles (arrows), reminiscent to the phenotype of *st8sia2* knockdowns. (C and F) In *ncam1b* knockdown embryos, pc axons cease their dorsally directed growth in a region where *ncam1b* expression in the midbrain usually starts (arrows) and only a few axons continue their growth toward the dorsal midline (arrowheads). (**G**) Quantification of growth pattern defects of the pc after knockdown of *ncam1a*, *ncam1b* or *st8sia2*. Three phenotypes of pc axon growth patterns are classified: "fasciculated", the pc forms a single fasciculated bundle (blue); "split bundle", the pc is separated into several bundles of medium thickness (red); "ceased growth", most axons of the pc stop their growth before reaching the midline (green). The midline is indicated by a dashed line. Significance was tested with a $k \times 2$ chi-square test after Brandt–Snedecor ($P < 0.001$). (**H**) Co-injection of mRNAs encoding for Ncam1a or Ncam1b rescues the phenotype induced by morpholino knockdown. Quantification as in (G).

pattern of axons in the pc in specific ways. Phenotypes resulting from knockdowns of either *ncam1a* or *ncam1b* are rescued by co-injection of *ncam1a* or *ncam1b* mRNAs, indicating the specificity of the effects (Figure 7H). Rescue is significant, though not complete, which results from the fact that the penetrance and distribution of injected mRNAs are reduced compared with those of the small morpholino molecules (Eisen and Smith 2008; Bill et al. 2009). Whereas the axons of the pc grow in one tightly fasciculated bundle in over 75% of control morpholino-injected embryos (arrow in Figure 7A; "fasciculated" phenotype in Figure 7G), knockdown of polysialyltransferase St8Sia2 causes the pc to split

up into several fasciculated axon bundles in 47% of the embryos (arrows in Figure 7D, "split bundle" phenotype in Figure 7G). Removal of Ncam1a practically reproduced the effects observed after polySia removal; 56% of the embryos show the "split bundle" phenotype under these conditions (Figure 7B, E and G). Separation of the pc into these bundles is restricted to more medial regions (arrows in Figure 7B, E and D). Removal of Ncam1b causes a different phenotype: in 54% of the embryos most axons of the pc seem to cease their dorsally directed growth in a region where Ncam1b expression in the midbrain usually starts (arrows in Figure 7C and F; "ceased growth" phenotype Figure 7G). Only a few, single axons continue their growth toward and across the dorsal midline (arrowheads in Figure 7C and F). In *ncam1b* knockdowns, we neither found an obvious reduction in cells in the nuclei from which the pc emanates nor a reduction in the thickness of the pc before it reaches the midbrain region which normally expresses *ncam1b*. Thus, we conclude that Ncam1b is required neither for the specification of pc neurons nor for the initiation of axon outgrowth. The most obvious explanation for the observed pc growth pattern is that Ncam1b on brain cell bodies is a prerequisite for pc axons to continue growing toward the midline in the dorsal midbrain. The diverse functional roles of Ncam1a and Ncam1b during the development of the pc clearly indicate a subfunctionalization after gene duplication. Whereas polySia on Ncam1a is involved in the fasciculated growth pattern of axons in the pc, Ncam1b serves as an instructive guidance cue for the dorso-medial directed growth of these axons. This feature of Ncam1b might be independent of polysialylation, as the removal of polySia—despite inducing the "split bundle" phenotype—does not inhibit the dorsally directed outgrowth of pc axons.

Besides the pc, spinal motor axons are a major population of axons which carry polySia. Here, Ncam1b was determined as the predominant polysialylated cell adhesion molecule (Figure 4). Whereas the removal of polySia was found to have no effect on the motor axon growth pattern (Marx et al. 2001, 2007), morpholino knockdown of *ncam1b* induces obvious morphological defects. The ventrally projecting motor axons extend irregularly, follow a staggered path and show ectopic branching rather than following the straight route observed in controls (Figure 8). The phenotype after *ncam1a* knockdown is less severe, as *ncam1a* is less prominently expressed in the spinal motor axons (Figure 8). Ncam1b is, thus, required in spinal motor axons for correct extension into their target region. The morphant phenotypes resulting from morpholino-induced knockdowns of *ncam1a* and *ncam1b* clearly show that the duplicated polySia carriers in teleost fish perform diverse and intricately regulated functions in axon growth and guidance.

Discussion

By combining bioinformatics and phylogenetic analyses, molecular developmental techniques and loss-of-function experiments, we determined the evolutionary origin of the members of the *ncam* gene family in teleost fishes and analyzed their activity in the developing zebrafish embryo.

Our analyses identify the fish *ncam1a* and *ncam1b* genes as duplicated paralogs shared by teleost fishes in contrast to a single *NCAM1* gene found in all tetrapod species analyzed. The duplication of the ancestral *NCAM1* gene could have resulted from the ray-finned fish-specific genome duplication event which took place ~350 million years ago (Amores et al. 1998; Christoffels et al. 2004; Jaillon et al. 2004; Vandepoele et al. 2004; Meyer and Van de Peer 2005). Following duplication, the most probable fate for a pair of duplicated genes is non-functionalization of one copy by

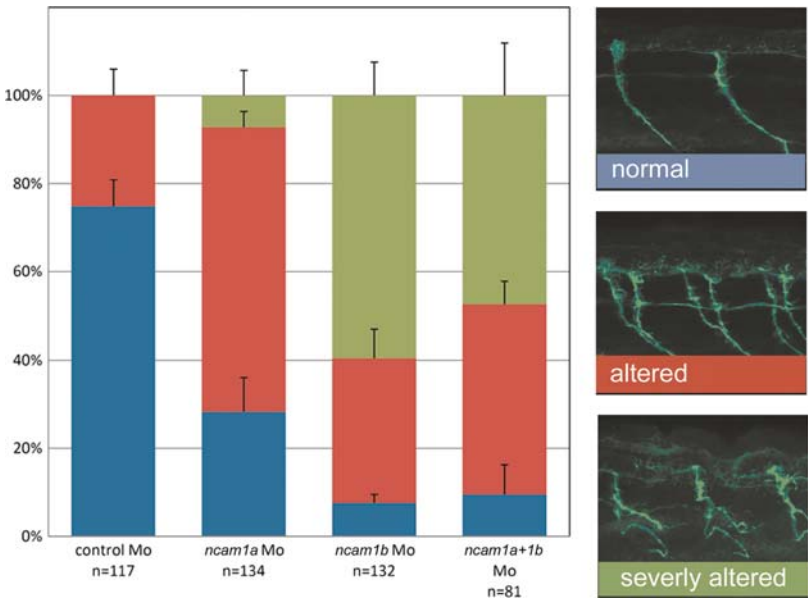

Fig. 8. Knockdown of *ncam1b* affects the growth of spinal motor axons. Wild-type embryos were injected at the 1-cell stage with the indicated morpholinos, and embryos were fixed at 36 hpf and immunostained for Tag-1 to visualize axons. To quantify growth pattern defects after knockdown, three phenotypes of axon growth patterns are classified as depicted in the right panel; shown are lateral views of the trunk region, rostral to the left.

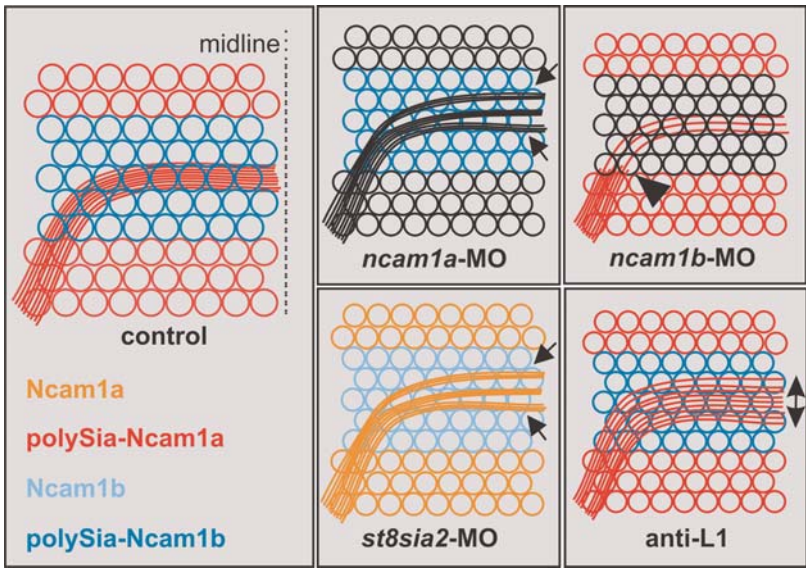

Fig. 9. Summary of Ncam1a, Ncam1b and polySia expression in the dorsal midbrain and the growth pattern of the pc after various knockdowns. Axons of the pc express only polySia-Ncam1a and grow as a single fasciculated bundle in a region of the midbrain with neuronal cell bodies expressing mainly polySia-Ncam1b. More rostral and more caudal regions of the midbrain express polySia-Ncam1a but not Ncam1b. Knockdown of either *ncam1a* or *st8sia2*, to remove polySia, causes axons of the pc to split into several fasciculated bundles (arrows). In contrast, blocking L1 with antibodies results in a defasciculation of the pc (double-headed arrow). Knockdown of *ncam1b* causes pc axons to cease their dorsally directed growth in a region where *ncam1b* expression in the midbrain usually starts (arrowhead).

accumulation of deleterious mutations under relaxed selective constrains (Ohno 1970). Two scenarios have been suggested to explain the retaining of both copies of a duplicated gene. One of the duplicated genes could have acquired a new beneficial function, which is then positively selected (neofunctionalization), whereas the other one retains an ancestral function (Ohno 1970). Alternatively, to explain the grade and endurance of retention of duplicated genes in genomes, the "duplication, degeneration and complementation model" proposes subfunctionalization, i.e. partitioning of ancestral functions between copies, as the outcome of duplication (Force et al. 1999). The combination of both scenarios (subfunctionalization followed by neofunctionalization) was also proposed (He and Zhang 2005; Lynch and Force 2009). Almost all identified duplicated gene pairs in fish genomes have diverged in spatial and/or temporal expression during embryogenesis and about a quarter of them diverged in functions by acquisition of novel protein domains or changes in the subcellular localization of gene products (Kassahn et al. 2009). Here, we report a further example of a pair of duplicated genes involved in the development of the CNS which underwent subfunctionalization.

The single NCAM1 of tetrapods has so far been regarded as the most prominent polysialylated protein in animals (Bonfanti 2006; Gascon et al. 2007, 2010; Hildebrandt et al. 2010). By a combination of morpholino knockdown and whole-mount immunostaining with specific antibodies, we here demonstrate that teleost fish express two Ncam1 paralogs which both carry polySia. In the developmental stages analyzed in this study, the expression pattern of *ncam1b* matches the expression pattern of polySia after *ncam1a* knockdown indicating that there are no prominent cell populations expressing non-polysialylated Ncam1b. This indicates that polysialylation may be an equally important post-translational modification of both teleost Ncam paralogs. Morpholino knockdown of *st8sia2* results in a reduction in polySia to levels below the detection limit, suggesting that polySia formation in early zebrafish development exclusively depends on St8Sia2. The second polysialyltransferase encoded by the zebrafish genome, St8Sia4, is expressed in later developmental stages, rather weakly and only in small cell populations (Marx et al. 2007; Rieger et al. 2008; Chang et al. 2009). By ectopic co-expression in zebrafish, we have, however, shown that St8Sia4, like St8Sia2, is able to add polySia to Ncam1a and Ncam1b. St8Sia4 may participate in regulating the polySia state of Ncam1a and/or Ncam1b in particular cell populations in later larval stages or in adult fish. The feature of polysialylation has, accordingly, been retained by both teleost Ncam paralogs.

Several groups of neurons in the CNS express Ncam1a or Ncam1b exclusively, which suggests a functional diversification of these cell adhesion molecules. One example for a cell population highly specialized in this regard is the floorplate of the spinal cord which is dominated by Ncam1a, whereas Ncam1b shows a somewhat stronger expression in the rest of the spinal cord. Commissural axons that cross the floorplate do not express Ncam1a but they require polySia-Ncam1a on the floorplate as a molecular cue for growing toward the contralateral side (Marx et al. 2001). The interaction partner on these axons for polySia on floorplate cells has yet to be identified. The effect of *ncam1b* knockdown on the growth pattern of commissural hindbrain axons was not studied herein, since these axons and the floorplate do not express detectable amounts of Ncam1b.

The situation is different for axons of the pc. These axons cross dorsally at the junction of the forebrain and the midbrain in a fasciculated bundle. As summarized in Figure 9, axons of

the pc express polySia-Ncam1a in addition to the Ig-superfamily member L1 (Weiland et al. 1997). These axons project to the contralateral side by growing in contact with cells expressing mainly polySia-Ncam1b. Removal of polySia from both the pc axons and their environment by either injection of the polySia degrading enzyme EndoN (Marx et al. 2001) or morpholino knockdown of the polySia synthesizing enzyme St8Sia2 (Marx et al. 2007) induces a split of the pc into several tightly fasciculated bundles of medium thickness (Figure 9) crossing the midline. This phenotype contrasts the effects of blocking L1 by functional antibodies (Weiland et al. 1997) which cause the pc to completely defasciculate and form a single but thicker bundle of loosely associated axons which cross the midline (Figure 9). We have interpreted the polySia-negative phenotype to result from an increase in environmental attraction which causes several single growth cones of pioneering axons to steer toward the dorsal midline on a more deviated path (Marx et al. 2001). Follower axons then build fascicles with any of the pioneering axons. Morpholino knockdown of *ncam1a* reduces polySia immunoreactivity on the pc axons only, but not in their environment. Since this induces the same growth pattern as the removal of total polySia (Figure 9), we conclude that polySia on commissural axons (attached to Ncam1a) but not in their environment (attached to Ncam1b) is required for preventing the pc to split into several bundles. While the role of Ncam1a in formation of the pc seems to be reduced to that of a carrier of polySia on the pc axons, Ncam1b has a different function. In *ncam1b* morpholino knock-down embryos, most pc axons cease their growth in a lateral position and never reach the dorsal brain surface (Figure 9). Only a few axons cross, either as single axons or in small bundles. Thus, Ncam1b seems to be required in the environment of the pc axons to provide supportive growth conditions which enable the axons to cross the brain hemispheres. In contrast, polySia attached to Ncam1b in the environment seems not to be necessary because polySia removal does not prevent pc axons from growing toward the midline (Figure 9). The nature of the axonal ligand for Ncam1b is not known. However, our experiments suggest that it is neither Ncam1b, since it is not found on axons of the pc, nor Ncam1a, since the removal of Ncam1a from the axons does not lead to a growth arrest. In summary, a well-balanced interplay of at least three members of the Ig superfamily and their glycosylation by polysialylation seems to be involved in proper guidance of pc axons toward the dorsal midline. Similar observations have been made for axons of the anterior commissure in zebrafish embryos, where the interactions of L1 and Semaphorin3D regulate the fasciculation pattern (Wolman et al. 2007). Whereas axons of the pc require Ncam1b in their surroundings for correct extension, spinal motor neurons dependent on axonally expressed Ncam1b for regular growth pattern. Both situations are similar as far as they seem not to be dependent on polySia-attached Ncam1b.

In conclusion, *ncam1a* and *ncam1b* have undergone substantial functional diversification after having arisen from a common ancestor by genome duplication. Differences on the molecular level are most obvious in the cytoplasmic domains of the proteins, a region considerably smaller in Ncam1a than in Ncam1b. The cytoplasmic domain of Ncam1b contains an amino acid stretch which is encoded by a homolog of mouse *ncam1* exon 18 and is not found Ncam1a. As the cytoplasmic domain contains binding sites for various intracellular signal adaptor and effector proteins (Büttner et al. 2005; Maness and Schachner 2007; Cassens et al. 2010; Kleene et al. 2010), these differences might have conferred an important functional diversification of *ncam1a* and *ncam1b*. On the cellular level, diversification is furthermore realized by distinct expression domains. These findings clearly argue for a subfunctionalization of *ncam1a* and *ncam1b*; convincing evidence for a neofunctionalization is yet elusive. Gene duplication in the teleost lineage has created the basis for intricately fine-tuning the regulation of cell adhesion and axon guidance phenomena by varying the spatio-temporal expression of two NCAM paralogs and two polysialyltransferases. In more general, our data highlight the suitability of zebrafish as a non-mammalian model (i) to study evolutionary mechanisms after gene duplication and (ii) to elucidate the diverse functions of neuronal cell adhesion molecules and their modification by polySia in the nervous system.

Materials and methods

Animal care and maintenance

Wild-type and golden strains of the zebrafish, *Danio rerio*, were obtained from the University of Konstanz. Embryos were collected by natural spawning and raised at 28.5°C in E3 medium (Haffter et al. 1996) containing 0.02% Methylene blue. Developmental stages were characterized by the criteria of Kimmel et al. (1995). For immunostaining, 0.003% 1-phenyl-2-thiourea was added to the E3-incubation medium 24 hpf. Embryos were anesthetized and then subjected to fixation with 4% paraformaldehyde.

Phylogenetic and genomic analyses

Complete coding regions of the 140 kDa isoform of *ncam1a* and of *ncam1b* were obtained by reverse transcription of total RNA (24 and 36 hpf embryos) and subsequent polymerase chain reaction (PCR) amplification. They were cloned into plasmid vector (pCRII-Blunt-TOPO®; Invitrogen, Carlsbad, CA) and confirmed by sequencing (LCG Genomics). GenBank accession numbers are: *ncam1a*, HM467818; *ncam1b*, HM467819. Previously determined sequences of the 140 kDa isoform of Ncam1a (NCAM), Ncam1b (PCAM) and Ncam2 (OCAM) of zebra fish (*D. rerio*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridis*) and fugu fish (*Takifugu rubripes*) were downloaded from the GenBank (www.ncbi.nlm.nih.gov) and Ensembl (http://www.ensembl.org) databases. Identities of obtained sequences were tested by reciprocal tBLAST searches for the best hit. Amino acid sequences were aligned using the ClustalW algorithm implemented in the MEGA version 4 (Tamura et al. 2007). NCAM1 and NCAM2 coding sequences of human, mouse, chicken and frog were included as outgroups for each gene. Phylogenetic analyses were conducted under neighbor joining (NJ), minimum evolution (ME) and maximum parsimony (MP) algorithms implemented

in MEGA version 4 (Tamura et al. 2007). For the NJ and ME analyses, the maximum composite likelihood model of substitutions was chosen, gaps and missing data were deleted pairwise and all codon positions were analyzed. For the MP analyses, we used the close neighbor interchange (CNI) search method with the initial tree by random addition (10 replicates). Gaps and missing data were completely deleted under MP. Reliability of all obtained tree topologies were tested by the bootstrap method with 1000 replicates (Felsenstein 1985).

Morpholino injections

To knockdown the expression of cell adhesion molecules Ncam1a and Ncam1b in zebrafish embryos, we used a morpholino antisense approach (Nasevicius and Ekker 2000). The following morpholinos (Gene Tools, Philomath, OR) were applied: *ncam1a* morpholino 1, 5′-TTCCGTGTAGAATAGG TAGAGTTGG-3′; *ncam1a* morpholino 2, 5′-TGAGATCCCG TATTTGAAGCATTGC-3′; *ncam1b* morpholino 1, 5′-AGATTATCGCCTTGGTCGGAAACAT-3′; *ncam1b* morpholino 2, 5′-GTTTACTGTTTGTTTTTGCCTTCCG-3′. *St8Sia2* was knocked down as described previously (Marx et al. 2007). The standard control morpholino (Gene Tools) was used as the injection control. Morpholinos were injected in concentrations of 0.15–0.3 mM in 1× Danieau solution into 1–2-cell stage embryos. The efficiency and the effects of the morpholinos were analyzed by immunostainings.

Immunohistochemistry

Zebrafish embryos were processed for whole-mount immunohistochemistry as described previously (Weiland et al. 1997; Marx et al. 2001). Ncam1a, Ncam1b or Ncam2 were visualized by using rabbit polyclonal antibodies (Miyasaka et al. 2005). Polyclonal antibody 704, which was generated by immunization with a mixture of polySia carrying membrane proteins from goldfish brain (Marx et al. 2001), was used to label both Ncam1a and Ncam1b. Axons were labeled with antibodies against zebrafish Tag-1 (polyclonal antibody 741, Lang et al. 2001; monoclonal antibody T8/9). PolySia was labeled with monoclonal antibody 735 (Frosch et al. 1985). The following secondary antibodies were used: Cy3-conjugated AffiniPure F $(ab')_2$ fragment donkey anti-mouse IgG (H + L) (1.4 µg/mL; Jackson ImmunoResearch, West Grove, PA), Cy3-conjugated AffiniPure F$(ab')_2$ fragment donkey anti-rabbit IgG (H + L) (1.5 µg/mL; Jackson ImmunoResearch), Alexa488-conjugated F$(ab')_2$ fragment of goat anti-rabbit IgG (H + L) (2 µg/mL; Invitrogen) and Alexa488-conjugated F$(ab')_2$ fragment of goat anti-mouse IgG (H + L) (2 µg/mL; Molecular Probes). After immunostaining, the yolk was removed and the embryos were embedded in Mowiol. Embryos were analyzed using a confocal microscope (LSM 510, Zeiss) equipped with a high aperture lens 40×/1.2W (C-Apochromat, Zeiss) and the appropriate lasers. Serial optical sections were flattened into projections and processed with PhotoshopCS2/9.0.2 software (Adobe).

PolySia quantification

As an affinity probe for the quantification of polySia, we used a fusion protein consisting of GFP and a catalytically inactive form of EndoN (Jokilammi et al. 2004). Whole mounts of morpholino-injected embryos were basically treated as in the immunohistochemistry protocol: they were incubated with the fusion protein (30 µg/mL) at 4°C over night, embedded in Mowiol and then analyzed by confocal laser scanning microscopy. Quantification of the fluorescence intensity in selected regions of the embryo was determined with the LSM Image Examiner software (Zeiss). Data were combined from three independent experiments, in which five specimen of each condition where analyzed. The intensity value of control morpholino-injected embryos was set to 100% in each experiment.

Ectopic protein expression

Ectopic expression of *ncam1a*, *ncam1b*, *st8sia2* and *st8sia4* in zebrafish embryo muscle was performed using the Tol2 transposable element (Kawakami et al. 2004; Urasaki et al. 2006). PCR products were cloned into plasmids of the Tol2 Kit (Chien Laboratory; Kwan et al. 2007) by *att* site-specific recombination-based cloning (MultiSite Gateway Technology, Invitrogen, Carlsbad, CA). By BP recombination, the 5′ entry vector was constructed from vector p5E-MCS and the zebrafish α-actin promoter (gift of M. Granato, U. Pennsylvania). Middle entry clones (pME) were constructed from pDONR221 and complete open reading frames of *ncam1a*, *ncam1b*, *st8sia2* and *st8sia4*. p3E was used as 3′ entry vector. 5′, middle and 3′ entry vectors were cloned by LR recombination into destination vector pDestTol2pA2 (Kwan et al. 2007). For transient expression, DNA of the resulting expression was injected into 1–2-cell stage embryos together with capped Tol2 transposase mRNA, which was generated from vector PCS2FA (Tol2 kit) by the mMessage mMachine system (Ambion, Austin, TX).

Rescue experiments

Full-length coding regions of *ncam1a* or *ncam1b* were used for rescue experiments. *Ncam1b* was cloned into vector pCS2-vector and five mismatches were generated at the morpholino-binding site by site-directed mutagenesis. *Ncam1a* was cloned into vector pcDNA3.1(+), and morpholino knockdown was performed with a 5′UTR morpholino. Capped RNA was transcribed from linearized templates using mMessage mMachine kit (Ambion). RNA was injected into 1–2-cell stage embryos in two different concentrations.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

CNS, central nervous system; EndoN, endosialidaseN; GFP, green fluorescent protein; hpf, hours post fertilization; Ig, immunoglobulin; ME, minimum evolution; MP, maximum parsimony; NCAM, neural cell adhesion molecule; NJ, neighbor joining; pc, posterior commissure; PCR, polymerase chain reaction; polySia, polysialic acid.

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