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Syntenin-a promotes spinal cord regeneration following injury in adult zebrafish

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Abstract

In contrast to mammals, adult zebrafish recover locomotor function after spinal cord injury, in part due to the capacity of the central nervous system to repair severed connections. To identify molecular cues that underlie regeneration, we conducted mRNA expression profiling and found that syntenin-a expression is upregulated in the adult zebrafish spinal cord caudal to the lesion site after injury. Syntenin is a scaffolding protein involved in mammalian cell adhesion and movement, axonal outgrowth, establishment of cell polarity, and protein trafficking. It could thus be expected to be involved in supporting regeneration in fish. Syntenin-a mRNA and protein are expressed in neurons, glia and newly generated neural cells, and upregulated caudal to the lesion site on days 6 and 11 following spinal cord injury. Treatment of spinal cord-injured fish with two different antisense morpholinos to knock down syntenin-a expression resulted in significant inhibition of locomotor recovery at 5 and 6 weeks after injury, when compared to control morpholino-treated fish. Knock-down of syntenin-a reduced regrowth of descending axons from brainstem neurons into the spinal cord caudal to the lesion site. These observations indicate that syntenin-a is involved in regeneration after traumatic insult to the central nervous system of adult zebrafish, potentially leading to novel insights into the cellular and molecular mechanisms that require activation in the regeneration-deficient mammalian central nervous system.

Introduction

Adult fish have the capacity for repair of central nervous system injury (Bernstein, 1964; Becker *et al.*, 1997, 1998, 2005). Use of the adult zebrafish model of spinal cord injury (SCI) to investigate spinal cord repair mechanisms has led to the identification of several regeneration-related genes, such as adhesion molecules L1.1 (Becker *et al.*, 1998) and tenascin-C (Yu *et al.*, 2011a), transcription factor sox11b (Guo *et al.*, 2011), cytokine fibroblast growth factor 2 (Goldshmit *et al.*, 2012), miRNA 133b (Yu *et al.*, 2011b), contactin-2 (Lin *et al.*, 2012) and major vault protein (Pan *et al.*, 2013). In adult zebrafish, successful regeneration depends on neuron-intrinsic properties and a permissive environment for a successful neuronal response to this environment (Becker *et al.*, 1998, 2005), including motoneuronal regeneration (Reimer *et al.*, 2008) and glial bridge formation (Goldshmit *et al.*, 2012). In characterising the mRNA expression profile of spinal cord cells caudal to the lesion site after SCI (Guo *et al.*, 2011), we observed upregulation of syntenin-a, the homologue of mammalian syntenin-1.

Syntenins belong to an expanding family of PDZ domain-containing scaffolding proteins (Grootjans *et al.*, 1997). Syntenin-1, originally identified as melanoma differentiation-associated gene-9 (mda-9; Lin *et al.*, 1996), was subsequently named syntenin following its recognition as a syndecan-binding protein (Grootjans *et al.*, 1997; Sarkar *et al.*, 2004). Syntenin-1 is widely expressed in mammalian cells (Zimmermann *et al.*, 2001), and is associated with receptor clustering, protein trafficking, cytoskeleton signaling (Grootjans *et al.*, 1997; Sarkar *et al.*, 2004, 2008; Yang & McCarthy, 2007; Beekman & Coffer, 2008) and metastatic potential through cytoskeletal reorganisation in various cancer cells (Koo *et al.*, 2002; Boukerche *et al.*, 2005; Hwangbo *et al.*, 2010, 2011). Thus, cytoskeletal reorganisation appears to be a major function of syntenin-1 in many cell types due to its ability to interact with a broad functional spectrum of molecules, such as syndecan, neurofascin, ephrin-B, src, ubiquitin, Unc51.1, Rab5 and Sox4 (Grootjans *et al.*, 1997; Koroll *et al.*, 2001; Tomoda *et al.*, 2004; Beekman & Coffer, 2008; Boukerche *et al.*, 2008; McClelland *et al.*, 2009; Okumura *et al.*, 2011; Rajesh *et al.*, 2011; Das *et al.*, 2012), resulting in distinct functional outcomes (Fig. S1A).

Syntenin-1 is widely distributed throughout the central nervous system and is associated with axon extension, dendritic protrusion, synapse formation and maturation (Zimmermann *et al.*, 2001; Beneyto & Meador-Woodruff, 2004; Ohno *et al.*, 2004; Tomoda *et al.*, 2004; Hirbec *et al.*, 2005; Ko *et al.*, 2006; McClelland *et al.*, 2009; Xu *et al.*, 2011) and oligodendroglial precursor migration (Chatterjee *et al.*, 2008). Recent reports reveal abundant expression of syntenin-1 in developing spinal cord (Jeon *et al.*, 2013). Similar to mammalian syntenin-1 and syntenin-2, zebrafish express syntenin-a and syntenin-b. Syntenin-a is the zebrafish homologue of syntenin-1

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(Fig. S1B), revealing epiboly progression (Lambaerts *et al.*, 2012). In this study, we established for the first time that syntenin-a is required for functional regeneration, as would be expected from its widely based cellular functions in mammalian cells. These observations may bear on our understanding of the functions of syntenin-a in mammals.

Materials and methods

SCI model

The protocols for the SCI model were based on those described earlier (Becker *et al.*, 1997; Guo *et al.*, 2011; Yu *et al.*, 2011a,b; Ma *et al.*, 2012). Briefly, male adult zebrafish (*Danio rerio*; body length >2.5 cm, age 6 months; Huiyuan Aquatic Animals Company, Shantou, Guangdong, China) were immersed in 0.033% aminobenzoic acid ethylmethylester (MS222; Sigma-Aldrich, St Louis, MO, USA) in phosphate-buffered saline, pH 7.3 (PBS) for 5 min to allow complete anaesthesia. A longitudinal incision was made to expose the vertebral column, and the spinal cord was cut completely between two vertebrae, 4 mm caudal to the brainstem–spinal cord junction. The sham-lesioned control was performed using the same procedures, but without SCI. Incisions were sealed with Histoacryl (B. Braun, Melsungen, Germany). The animal experiments were conducted under the approval of the Animal Ethics Committee of Shantou University Medical College. Fish were kept on a 14-h light and 10-h dark cycle at 28 °C.

Morpholino (MO) application

Two different syntenin-a anti-sense MOs (Syn-MOs; NM_212691; Syn-MO1, 5′-CCTCTAGCGATGGGTACAACGACAT-3′; Syn-MO2, 5′-TTCTGCTTTCAAACAAGCGACTTCT-3′; Gene Tools, Philomath, OR, USA) and a standard control MO (Con-MO; 5′-CCTCTTACCTCAGTTACAATTTATA-3′) were dissolved in Danieau solution [in mM: NaCl, 58; KCl, 0.7; $MgSO_4$, 0.4; $Ca(NO_3)_2$, 0.6; and HEPES, 5; pH 7.6] and soaked into small pieces of Gelfoam (Upjohn, Kalamazoo, MI, USA). The Gelfoam was allowed to dry and divided into smaller pieces containing 600 ng MO per piece. One dried Gelfoam piece was inserted into the lesion immediately after transection (Becker *et al.*, 2004).

Quantitative real-time polymerase chain reaction (qPCR)

Zebrafish were deeply anesthetised with MS222 at 4, 12 h, 6 or 11 days after SCI or sham injury ($n = 5$ per group), and killed. Spinal cord tissue 5 mm caudal to the lesion site was rapidly removed, immediately frozen on dry ice and stored at −80 °C until use.

Total RNA was extracted from frozen spinal cord samples using an EZgene™ Tissue RNA Miniprep Kit (Biomiga, San Diego, CA, USA) according to the manufacturer's instructions. Twenty nanograms of total RNA were reverse-transcribed using PrimeScript® RT Master Mix (Takara). For qPCR analysis, the following primers were used: syntenin-a, 5′-TTCACTTCTGCTCCAACC-3′ (forward) and 5′-GTGGCATTAGCAAACTGG-3′ (reverse); GAPDH, 5′-GTGTAGGCGTGGACTGTGGT-3′ (forward) and 5′-TGGGAGTCAACCAGGACAAATA-3′ (reverse). The qPCR was performed using SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) on an ABI PRISM 7300 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. Experiments were performed in triplicate. Single PCR products were confirmed by melting curves from qPCR. The results were normalised to GAPDH and analysed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

In situ hybridisation (ISH)

Zebrafish were deeply anesthetised with MS222 at 4, 12 h, 6 or 11 days after SCI or sham injury ($n = 4$ per group) and killed. Spinal cord tissue 5 mm caudal to the lesion site was rapidly removed and immediately fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C for 6 h, and then washed in PBS containing 15% sucrose for 8 h at 4 °C. Longitudinal frozen sections (16 μm) were prepared in a cryostat (LEICA CM 1850) and stored at −80 °C until use. Each slide contained a section from all time points in order to accurately compare the signal between the time points.

Non-radioactive ISH was performed as described (Bernhardt *et al.*, 1996; Becker *et al.*, 1998). DIG-labeled cRNA probes for syntenin-a (NM_212691) were generated by *in vitro* transcription using T7 and SP6 RNA polymerases, and represented bases 651–1059 of the coding sequence. Spinal cord sections were hybridised for 18 h at 55 °C after pre-hybridisation for 3 h at 55 °C. After high-stringency washing, sections were incubated with alkaline phosphatase-coupled anti-DIG Fab fragment antibodies (Roche) at 4 °C overnight, and color was developed with NBT/BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate; Roche). The specificity of hybridisation signals was determined by comparison with a sense cRNA probe under the same conditions.

Combined immunohistochemistry and ISH

We performed ISH and immunofluorescence in longitudinal frozen sections (20 μm thick for bromodeoxyuridine (BrdU) and 4 μm thick for other cell markers) of spinal cord, 5 mm caudal to the lesion site, taken at 6 days post-SCI or sham injury ($n = 3$ per group) to identify the cell types expressing syntenin-a mRNA. ISH was performed as described above. Following ISH, the sections were processed for immunofluorescence. Sections were rinsed in PBS, then blocked with 1% bovine serum albumin and 5% goat serum in PBS. Thereafter, sections were incubated with primary antibodies against HuC/D (1:50; Invitrogen/Molecular Probes), Islet-1 (1 : 100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), GFAP (1 : 100; Dako), or BrdU (1 : 200; Sigma-Aldrich) overnight at 4 °C. Sections were washed in PBS, incubated with secondary antibodies, including Cy3-conjugated goat anti-rabbit (1 : 500; Wuhan Boster Company, China) and FITC-Affinipure goat anti-mouse (1 : 500; Jackson Immunoresearch) antibodies, for 1.5 h at room temperature. Fluorescent images were observed using fluorescence microscopy (Axio Imager Z1; Zeiss) and photographed using AxioVision software. To better visualise fluorescent images superimposed on bright-field images, Adobe Photoshop software was used to convert the brightfield ISH image into a black-and-white negative image (gray scale image with black and white reversed) prior to merging with the fluorescent image. To label progenitor cells in S-phase, we intraperitoneally injected BrdU (Sigma-Aldrich) solution (2.5 mg/mL) in a volume of 50 μL twice at a 2-h interval as described, and killed the fish 2 h after the last injection (Adolf *et al.*, 2006; Guo *et al.*, 2011). Frozen sections were processed for 30 min in 2 N HCl at 37 °C before incubation with anti-BrdU antibodies.

Western blot analysis

The spinal cord samples were collected, as described above, 4, 12 h, 6 and 11 days after SCI, sham injury, MO application or

non-injury. Two pooled spinal cords per time point ($n = 4$) were lysed in RIPA buffer with 1% PMSF (Solarbio, Beijing, China) and centrifuged after homogenisation. Supernatants were transferred to clean tubes and protein concentrations were determined by a bicinchoninic acid protein assay kit (Solarbio). Proteins were separated on 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were incubated with antibodies for syntenin-1 (1 : 500; cat. no. GTX108391; GeneTex, Irvine, CA, USA) and tubulin (1 : 1000; Beyotime Biotechnology, Haimen, China), followed by incubation with HRP-conjugated secondary goat anti-rabbit for syntenin-1, and goat anti-mouse for tubulin (1 : 1500; Wuhan Boster Company, China). Signals were visualised using a BeyoECL Plus Western blotting detection system (Beyotime Biotechnology) and photographed via FluorChem Q software (Alpha Innotech's Gel and Western Imaging Systems, San Leandro, CA, USA). Quantitative band analysis was performed with ImageJ 1.44p software (Wayne Rasband, National Institutes of Health, USA) and the results were normalised to tubulin.

Locomotor analysis

We used a swim-tracking test to analyse locomotor recovery as described (Becker *et al.*, 2004; Guo *et al.*, 2011; Yu *et al.*, 2011a,b; Ma *et al.*, 2012). The total distance swum and duration of movement (during 5 min) of freely moving fish was measured weekly after SCI and MO application, using video tracking Ethovision XT software (Noldus, Wageningen, the Netherlands).

Retrograde tracing

At 6 weeks after spinal cord transection and MO application, a second transection was made at 4 mm caudal to the first spinal lesion site and the axonal tracer biocytin (Sigma-Aldrich) was applied immediately as described (Becker *et al.*, 2004; Yu *et al.*, 2011a,b; Ma *et al.*, 2012). After 24 h, the brain and brainstem were dissected and immediately fixed in 4% PFA in PBS at 4 °C for 12 h, then incubated in PBS containing 15% sucrose for 12 h at 4 °C. Transverse frozen sections (25 μm) were prepared in a cryostat ($n = 3$ fish per group). Sections were incubated with Cy3-conjugated streptavidin (Bioss, Beijing, China) overnight at 4 °C, and then labeled cells were counted under a Zeiss fluorescence microscope.

Anterograde tracing

At 6 weeks following the swim-tracking test, biocytin was applied to the second transection at the brainstem–spinal cord junction as described (Yu *et al.*, 2011a). Tissue 0–2 mm caudal to the original lesion site was taken and immediately fixed in 4% PFA in PBS at 4 °C for 12 h, then incubated in PBS containing 15% sucrose for 12 h at 4 °C, and transverse frozen sections (25 μm) were prepared in a cryostat. Sections were rinsed in PBS and blocked with 5% bovine serum albumin in PBS. Thereafter, the sections were incubated with primary antibody against synaptic vesicle protein 2 (SV2; 1 : 200; Developmental Studies Hybridoma Bank) overnight at 4 °C ($n = 3$ fish per group). Sections were washed in PBS, incubated with FITC-Affinipure goat anti-mouse secondary antibodies (1 : 500; Jackson Immunoresearch) for SV2, and Cy3-conjugated streptavidin for biocytin, for 1.5 h at room temperature. Newly formed synapses, defined as the point of colocalisation between biocytin and SV2, were observed under confocal microscopy (Olympus). For comparing regenerated axon profiles, three sections chosen from the corresponding spinal cord levels were evaluated. Selected sections per fish ($n = 3$ per group), at 0–2 mm caudal to the lesion site from each group, were taken and Cy3-fluorescence was photographed with a Zeiss fluorescence microscope. The percentage of threshold-stained area in the appropriate section area was measured using Image J 1.44p software (Yu *et al.*, 2011b). The experimenter was blinded to the treatment.

Antibody specificity

The syntenin-1 antibody used for Western blot analysis labeled two bands, but the predicted syntenin-a molecular mass (32 kDa) and the syntenin-a antibody instructions from the GeneTex datasheet (GTX108391), as well as the results comparing zebrafish samples with U87-MG glioblastoma cells, supported the low band as being the syntenin-a signal. The specificities of the antibodies for GFAP, Islet-1, HuC/D, BrdU and SV2 used for immunofluorescence staining have been extensively verified and used successfully in previous studies to specifically mark, respectively, glia, motoneurons, immature neurons, newly generated cells and synapses in the zebrafish (Reimer *et al.*, 2008; Hui *et al.*, 2010; Guo *et al.*, 2011; Yu *et al.*, 2011a; Goldshmit *et al.*, 2012; Pan *et al.*, 2013).

Statistical analysis

SPSS 17.0 software (IBM, USA) was used for data analysis. Data are presented as means ± SEM. We performed Student's *t*-test or one-way ANOVA, followed by Tukey's *post hoc* test when appropriate.

Results

Syntenin-a expression was upregulated in cells caudal to the SCI site

To determine whether syntenin-a is involved in regeneration, we first performed qPCR to investigate syntenin-a mRNA expression after SCI. According to the microarray results (GEO accession number, GSE20460; link for the GEO dataset is http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jbkhpcakoummudg&acc=GSE20460), levels of syntenin-a mRNA had increased by 11 days post-injury, with a 1.4 ± 0.1-fold change when compared to the sham-injured control taken at the same time point.

To confirm and quantify syntenin-a upregulation, we took spinal cord samples caudal to the lesion site at 4 and 12 h as well as at 6 and 11 days post-SCI, and performed qPCR. These time points represent the acute and the more chronic response phases of the spinal cord to the injury (Guo *et al.*, 2011). qPCR analysis showed that syntenin-a mRNA levels were upregulated 1.5- to 2-fold at 6 and 11 days after SCI compared with sham-injured and untreated controls. Expression peaked at 6 days, with a 2.1 ± 0.5-fold increase in expression ($n = 5$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$), and then decreased to 1.5 ± 0.2-fold at 11 days post-SCI ($n = 5$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$) compared with the sham-injured controls (Fig. 1A). No significant change in syntenin-a mRNA was seen in the acute phase response to SCI (4 and 12 h).

We then measured syntenin-a protein levels at same time points by Western blot analysis. In Western blots, syntenin-a antibody reacted with a band of ~34 kDa molecular mass, identical to the theoretical molecular mass of zebrafish syntenin-a and similar to the molecular weight of the band detected in human U87-MG glioblastoma cells (Fig. 1B). Western blot analysis showed that syntenin-a

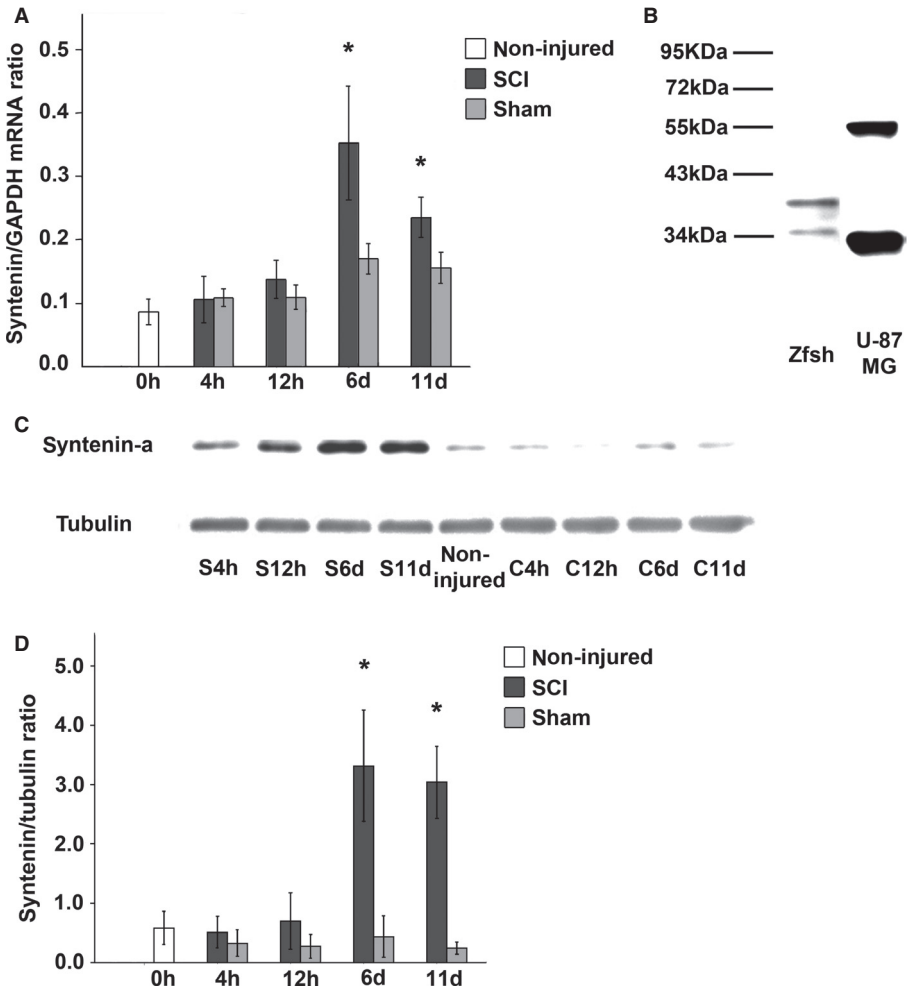

FIG. 1. Syntenin-a expression was upregulated post-SCI in the spinal cord caudal to the lesion site. (A) qPCR analysis showing that syntenin-a mRNA levels were upregulated at 6 and 11 days post-SCI compared to non-injured and sham-injured controls, and at 4 h and 12 h post-SCI. (B) Western blot analysis of syntenin-a in zebrafish spinal cord and U87-MG glioblastoma cells. Bands with an apparent molecular mass of ~34 kDa correspond to the reported value for syntenin-1. (C) Western blot analysis of syntenin-a in the spinal cord of non-injured fish, and spinal cord caudal to the lesion site in sham-injured fish, and fish at 4 and 12 h as well as 6 and 11 days post-SCI. S4 h–S11d indicate the time post-SCI; C4 h–C11d indicate the time after sham injury. (D) Quantitation of syntenin-a/tubulin ratios as estimated by Western blot analysis (C) showing that syntenin-a immunoreactivity was increased at 6 and 11 days post-SCI compared to non-injured and sham-injured controls and at 4 and 12 h post-SCI. Values are expressed as means ± SEM; $*P < 0.05$, one-way ANOVA with Tukey's *post hoc* test.

protein levels were increased 7.6 ± 1.1- and 12.5 ± 1.2-fold at 6 and 11 days post-SCI ($n = 4$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$), respectively, compared with sham-injured controls (Fig. 1C and D), and in temporal agreement with the qPCR results.

In order to determine the cellular expression patterns of syntenin-a in the spinal cord after SCI, we performed ISH on longitudinal sections of the spinal cord caudal to the lesion site. Similar to our qPCR results, syntenin-a expression was elevated at 6 and 11 days post-SCI, compared with sham-injured (Fig. 2A) and non-injured (Fig. 2C) controls, as well as at 4 and 12 h post-SCI. In non-injured and sham-injured zebrafish, as well as in zebrafish at the early phase of recovery (4 h and 12 h) post-SCI, syntenin-a was mainly expressed with moderate signals in neuron-like cells and with weak signals in glia-like and ependymal cells. However, in the chronic phase spinal cord (6 and 11 days, and particularly at 6 days post-SCI), syntenin-a was strongly expressed in both neuron- and glia-like cells located in and around the central canal and in the white matter. At 6 days post-SCI, there was a dramatic increase in numbers of syntenin-a-immunopositive cells immediately adjacent to the lesion site (Fig. 2B). No specific signals were detected in spinal cord sections when hybridised with a syntenin-a sense mRNA probe (Fig. 2D). These results show that syntenin-a expression is upregulated in the chronic phase post-SCI.

Identification of cell types expressing syntenin-a

To determine the cell types up-regulating syntenin-a expression we combined syntenin-a ISH and immunofluorescence for neural cell or glial cell markers caudal to the lesion site at 6 days post-SCI or sham injury (Figs 3–5). Syntenin-a mRNA was expressed in neurons positive for the motoneuronal marker Islet-1 (Fig. 4Aa–c), neurons expressing the immature neuronal marker HuC/D (Fig. 4Ba–c) and GFAP-positive glial cells (Fig. 3). Newly generated cells, as judged by BrdU pulse labeling, were also positive for syntenin-a mRNA (Fig. 5). In the sham-injured fish, syntenin-a mRNA was mainly expressed in ependymal cells and neurons, including motoneurons and immature neurons (Fig. 4A, B and Ca

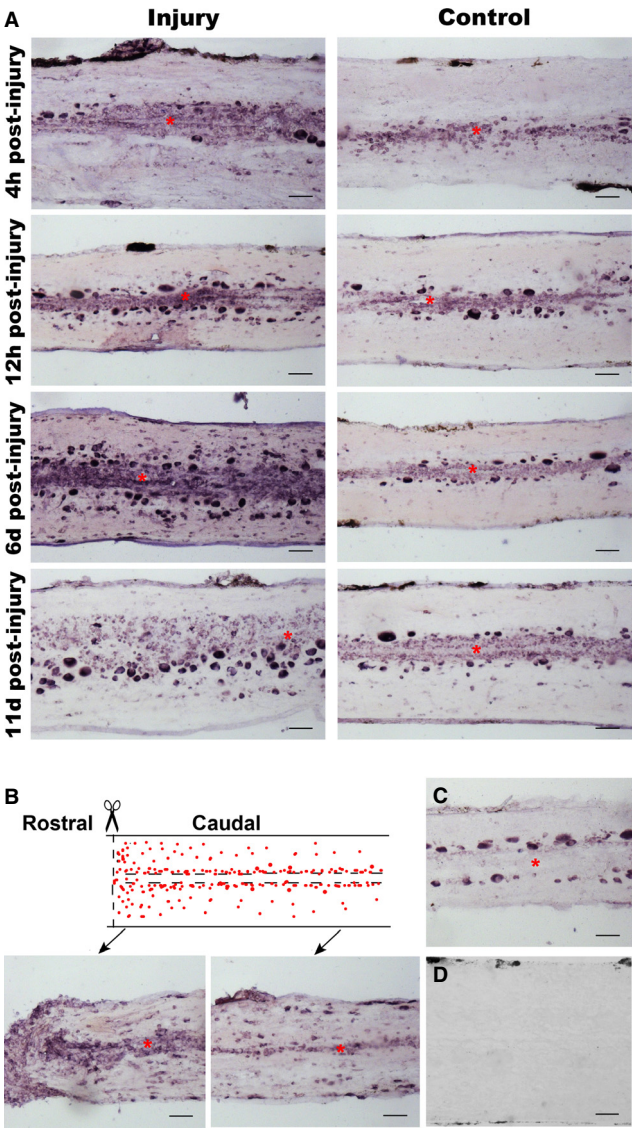

FIG. 2. ISH for syntenin-a in spinal cord at different time-points post-SCI. (A) Longitudinal sections of spinal cord caudal to the lesion site at 4 and 12 h as well as at 6 and 11 days post-SCI were hybridised with a syntenin-a anti-sense probe. Hybridisation signals were higher at 6 and 11 days compared to corresponding sham-injured controls. Particularly at 6 days post-SCI, strong signals were seen in neuron- and glia-like cells located in and around the central canal and in white matter, compared with sham-injured controls showing lower signals in neuron-like cells and weak signals in glia-like cells and ependymal cells. (B) At 6 days post-SCI, syntenin-a-positive cells showed a more pronounced increase immediately adjacent to the lesion site. (C) Syntenin-a mRNA was detected in the non-injured spinal cord in neuron-like cells and a few glia-like and ependymal cells. (D) Syntenin-a sense mRNA probe did not yield any signal. The lesion site is situated on the left side of all images; *indicates the central canal. Scale bars, 50 μm.

and b), and some GFAP-positive cells close to the central canal (Figs 3A–D and 4, Ca and b). At 6 days post-SCI, as well as in neurons, increased levels of syntenin-a mRNA were detected in a higher percentage of GFAP-positive cells located at and around the central canal (Figs 3E–H and 4, Da and b) compared to the sham-injured fish. We found that all HuC/D-positive cells expressed syntenin-a, and that HuC/D-negative, GFAP-positive cells also express syntenin-a 6 days after sham injury or post-SCI (Fig. 4C and D). We also found that some BrdU-positive cells expressed syntenin-a mRNA at 6 days post-SCI along the central canal site and in the region surrounding the central canal (Fig. 5E–H) while fewer BrdU-positive cells expressed syntenin-a mRNA along the central canal at 6 days after sham injury (Fig. 5A–D). Adjacent to the lesion site at 6 days post-SCI, several GFAP-positive glial cells co-expressing syntenin-a appeared in an elongated bipolar form reminiscent of ependymoradial glial cells (Fig. 4, Ea and b). These data indicate that syntenin-a is located in different cell types in the spinal cord and that expression is increased in the chronic phase post-SCI.

Syntenin-a promoted locomotor recovery

To investigate whether upregulation of syntenin-a expression is relevant to the recovery of locomotor function we performed swim-tracking analysis. At 5 weeks post-SCI, the total distance moved by fish treated with two independent Syn-MOs was reduced (Syn-MO1, 510.5 ± 153 cm in 5 min, $n = 12$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$; Syn-MO2, 666.6 ± 121.6 cm in 5 min, $n = 8$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$) compared to the Con-MO (1239.3 ± 110.9 cm in 5 min, $n = 10$) group, with the swim performance of Con-MO being similar to that of non-injured fish. Consistently, the duration of movement by fish treated with two independent Syn-MOs was reduced (Syn-MO1, 99.3 ± 33.3 s in 5 min, $n = 12$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$; Syn-MO2, 110.9 ± 34.7 s in 5 min, $n = 8$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$) compared to the Con-MO (233.2 ± 26.6 s in 5 min, $n = 10$) group. Also at 6 weeks post-SCI, the total distance moved and duration of movement by fish treated with Syn-MO1 was reduced (total distance, 601.4 ± 161.8 cm in 5 min, $n = 12$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$; duration of movement, 109.1 ± 33.2 s in 5 min, $n = 12$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$) compared to the Con-MO (total distance, 1212.4 ± 124.5 cm in 5 min, $n = 10$; duration of movement, 238.5 ± 26.2 s in 5 min, $n = 10$) group (Fig. 6A). However, we did not observe a significant reduction in the Syn-MO2 group in swim capacity, most likely resulting from a less efficient knock-down of translation when compared to MO1. The inhibition of syntenin-a expression by MO1 and MO2 was determined by Western blot analysis at 11 days after SCI and MO treatment. We confirmed that both Syn-MO1 and Syn-MO2 reduced syntenin-a protein levels, compared with the Con-MO, by 63% ($n = 4$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$) and 51% ($n = 4$, one-way ANOVA with Tukey's *post hoc* test, $P < 0.05$), respectively (Fig. 7A and B). These results indicate that syntenin-a function is important for locomotor recovery.

Syntenin-a was associated with axonal regrowth

By 6 weeks post-SCI, the spinal cord of fish treated with Con-MO had returned to almost normal appearance in tissue density at the lesion site. In contrast, the tissue density of the spinal cord remained reduced compared to the equivalent non-injured thoracic segment at 6 weeks post-SCI and Syn-MO1 treatment (Fig. 6B).

To determine the histological basis of the effect of Syn-MO treatment in locomotor recovery, retrograde tracing was performed at 6 weeks post-SCI immediately after the swim tracking analysis. The number of retrogradely labeled neurons in the nucleus of the medial longitudinal fascicle (NMLF) was significantly reduced in fish treated with Syn-MO1 (9.3 ± 3.5, $n = 3$, Student's *t*-test, $P < 0.05$) compared with Con-MO-treated fish (27.7 ± 4.3, $n = 3$; Fig. 6C and E). This result is consistent with previous studies showing that

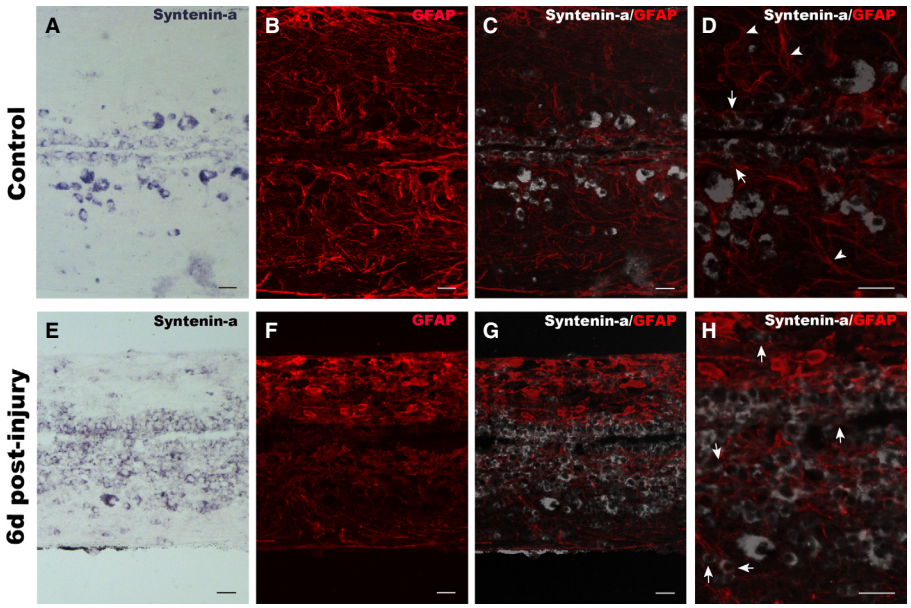

FIG. 3. Combined immunohistochemistry and ISH in longitudinal sections of spinal cord caudal to the lesion site at 6 days post-SCI or after sham injury shows syntenin-a mRNA expression in GFAP-positive glial cells. (A–D) Six days after sham injury, (A) syntenin-a mRNA is expressed in (B) some GFAP-positive cells close to the central canal; (C) merge of A and B; (D) Higher magnification of C. Arrows indicate co-localisation and arrowheads indicate GFAP-positive cells that do not express syntenin-a. (E–H) At 6 days post-SCI, (E) increased levels of syntenin-a mRNA are detectable in (F) more GFAP-positive cells at and around the central canal; (G) merge of E and F; (H) higher magnification of G. Arrows indicate co-localisation. Scale bars, 20 μm.

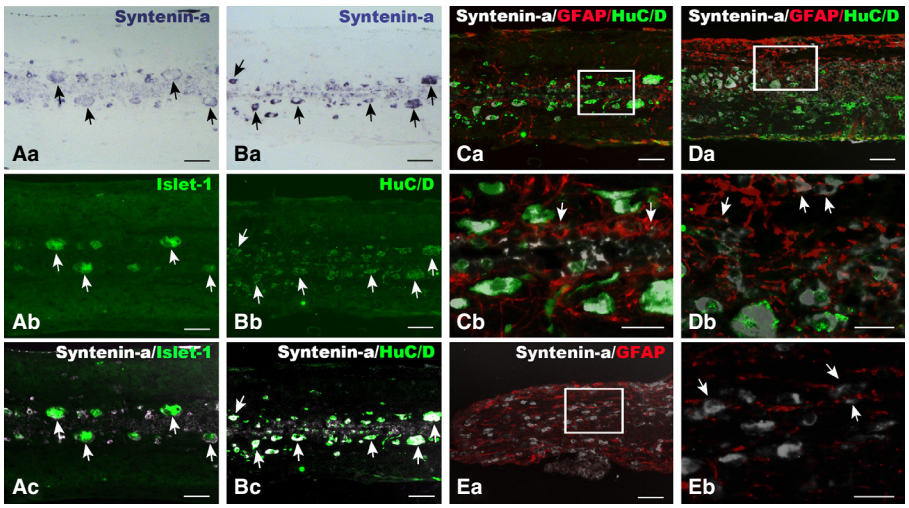

FIG. 4. Combined immunohistochemistry and ISH in longitudinal sections of spinal cords caudal to the lesion site at 6 days post-SCI or after sham injury showed syntenin-a mRNA expression in neurons and glial cells. Syntenin-a was expressed in neurons positive for the motoneuronal marker Islet-1 and the neuronal marker HuC/D for immature neurons. (Aa and Ba) Syntenin-a; (Ab), Islet-a; (Ac), merge of Aa and Ab; (Bb), HuC/D; (Bc), merge of Ba and Bb. Arrows indicate co-localisation. Syntenin-a was expressed not only in all HuC/D-positive cells but also in HuC/D-negative, GFAP-positive cells (arrows in Cb and Db) 6 days after sham injury (Ca–b) or post-SCI (Da–b). Cb and Db show higher magnifications of the boxed areas in Ca and Da, respectively. At the lesion site at 6 days post-SCI, many GFAP-positive glial cells appeared in an elongated bipolar form and some GFAP-positive cells expressed syntenin-a (arrows in Eb). Eb, higher magnification of boxed area in Ea. Scale bars, 20 μm (Cb, Db and Eb), 50 μm (A, B, Ca, Da and Ea).

the number of brainstem neurons with regenerated axons correlates with the recovery of locomotor function of individual fish (Becker *et al.*, 1997, 2004).

Syntenin-a expression was associated with synapse formation

Anterograde tracing was also performed to investigate whether syntenin-a is involved in synapse formation of regenerating axon terminals caudal to the lesion site. Newly formed synapses, represented by neurons co-labeled with the anterograde tracer biocytin and the synaptic marker SV2, were decreased in number in the Syn-MO1-treated fish (Fig. 8B and D) compared with Con-MO-treated fish (Fig. 8A and C). In agreement with this observation is the finding that the number of anterogradely labeled regrown axons labeled with biocytin were also decreased, to 45% in Syn-MO1-treated fish compared with Con-MO-treated fish ($n = 3$, Student's t-test, $P < 0.05$; Fig. 6D and F), consistent with the finding that fewer retrogradely labeled cell profiles were observed in the NMLF. We also failed to

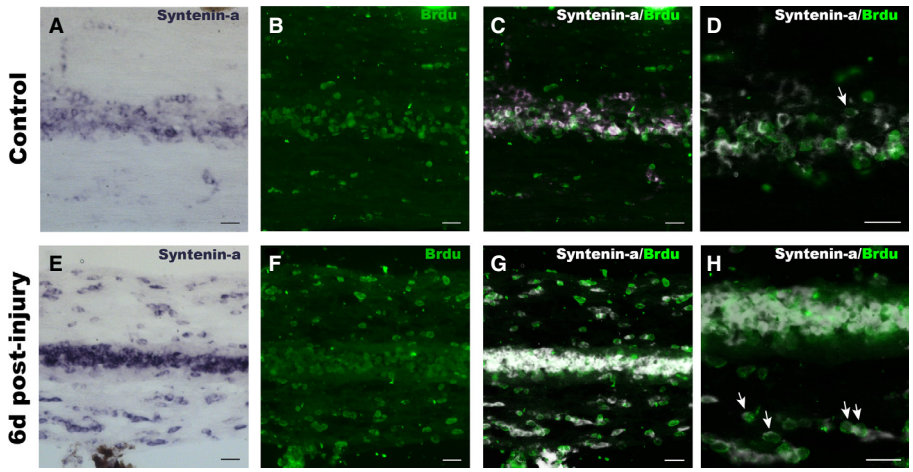

FIG. 5. Combined immunohistochemistry and ISH in longitudinal sections of spinal cords caudal to the lesion site at 6 days post-SCI or after sham injury shows newly generated BrdU-positive cells also expressing syntenin-a mRNA. (E–H) At 6 days post-SCI, (F) BrdU-positive cells along the central canal site and in the surrounding central canal also expressed (E) syntenin-a mRNA. (A–D) Sham-injured spinal cord showed (B) fewer BrdU-positive cells along the central canal and (A) fewer of these cells expressed syntenin-a mRNA. C, merge of A and B; G, merge of E and F; D and H show higher magnifications of C and G, respectively. Arrows indicate co-localisation of syntenin-a and BrdU. Scale bars, 20 μm.

detect caudal-to-rostral ascending axon regrowth that had crossed the lesion site in either Syn-MO-treated group.

Discussion

Treatment of SCI has remained problematic despite impressive efforts and successes to gain insights into the cellular and molecular mechanisms underlying the failures to regenerate after injury in adult mammals. To date, there is still no effective treatment that is accepted for therapy, although promising experimental techniques have been suggested from studies on animals, such as neutralising regeneration-inhibitory molecules, enhancing regeneration-conducive molecules and transplanting stem cells (for more recent reviews see Sahni & Kessler, 2010; Tohda & Kuboyama, 2011; Blesch *et al.*, 2012; Chew *et al.*, 2012; Gage, 2012; Pernet & Schwab, 2012; Sharma *et al.*, 2012). Regrowth and/or sprouting of severed axons and formation and/or remodeling of synapses are determined by the extrinsic glial environment and intrinsic neuronal competence (Bernhardt *et al.*, 1996; Becker *et al.*, 1998, 2005; Bernhardt, 1999; Yiu & He, 2006; Sun & He, 2010). As attempts to improve regrowth of axons in the adult via deletion of inhibitory molecules have so far failed to allow axons to regenerate over longer distances (Yiu & He, 2006; Sun & He, 2010), increased attention has been given to understanding the intrinsic regenerative ability of neurons. Syntenin-a may be one of many molecules that enhances this recovery, as it is expressed in neurons and neural precursor cells. Interestingly, syntenin-a is most prominently associated with recovery in the later, more chronic, phases of recovery from injury in adult zebrafish, suggesting that it preferentially affects the regrowth of axons and formation and/or remodeling of synaptic contacts than conditioning the tissue microenvironment. Nevertheless, syntenin-a mRNA and protein levels are upregulated caudal to the lesion site in the chronic phase after SCI. However, we have performed ISH in brain sections and did not find a difference in numbers of cells and intensity of signal between fish with no injury, sham injury and 6 days post-injury (data not shown). It is thus likely that intrinsic expression of syntenin-a in a non-injury situation is sufficient to ensure regrowth of axons after injury. It thus remains to be seen whether several beneficial cues, neuron-intrinsic or tissue environment (neuron-extrinsic) or both, are active in syntenin-a-mediated recovery from injury. Given that syntenin-a covers a wide spectrum of functions, neuron-intrinsic and -extrinsic features are likely to underlie the beneficial effects of this molecule in regeneration.

It is interesting in this context that, in the early phase of regeneration, glial cells accumulate at the lesion edge; this is followed by glial migration to the lesion site. The formation of a glial bridge permits axonal regeneration in zebrafish and enables axons to traverse the lesion site in a caudal direction (Zukor *et al.*, 2011; Goldshmit *et al.*, 2012). Our results reveal that syntenin-a is upregulated at 6 and 11 days post-SCI, consistent with the phase of glial accumulation and glial bridge formation. In addition, syntenin-a expression also accumulates in glial cells immediately adjacent to the lesion site at 6 days post-SCI, further supporting the view that syntenin-a participates in glial bridge formation and/or changes in glial morphology.

In the early development of zebrafish, the syntenin–syndecan–PIP2–Arf6 complex affects cell movements (Lambaerts *et al.*, 2012), and it is thus possible that this complex is also associated with newly generated neural cell and immature or mature glial cell migration in recovery of adult zebrafish. Also, a correlation between syntenin-1 and tyrosine kinase Src or focal adhesion kinase expression occurs in human melanoma cells, where syntenin-1 is associated with anchorage-independent growth, cell migration and metastasis of melanoma cells *in vitro* and *in vivo* (Boukerche *et al.*, 2008, 2010).

It is also noteworthy that some newly generated cells in the generative zone around the central canal express syntenin-a in the early and late phases of recovery. Syntenin-1 has been reported to be upregulated in stem cell clusters of human interfollicular epidermis (Estrach *et al.*, 2007). In zebrafish, syntenin transcripts are maternally supplied and expression of syntenin persists during gastrulation (Lambaerts *et al.*, 2012). These results show that syntenin-a is expressed in early ontogenetic development, implicating this molecule in important cellular functions providing cell motility and/or orientation. It is therefore striking that these functions appear to be recapitulated at least to some extent in regeneration of adult zebrafish, when some developmental events appear to provide an advantage for regeneration. Consistently, expression decreases in the adult mammalian central nervous system when maturation is completed,

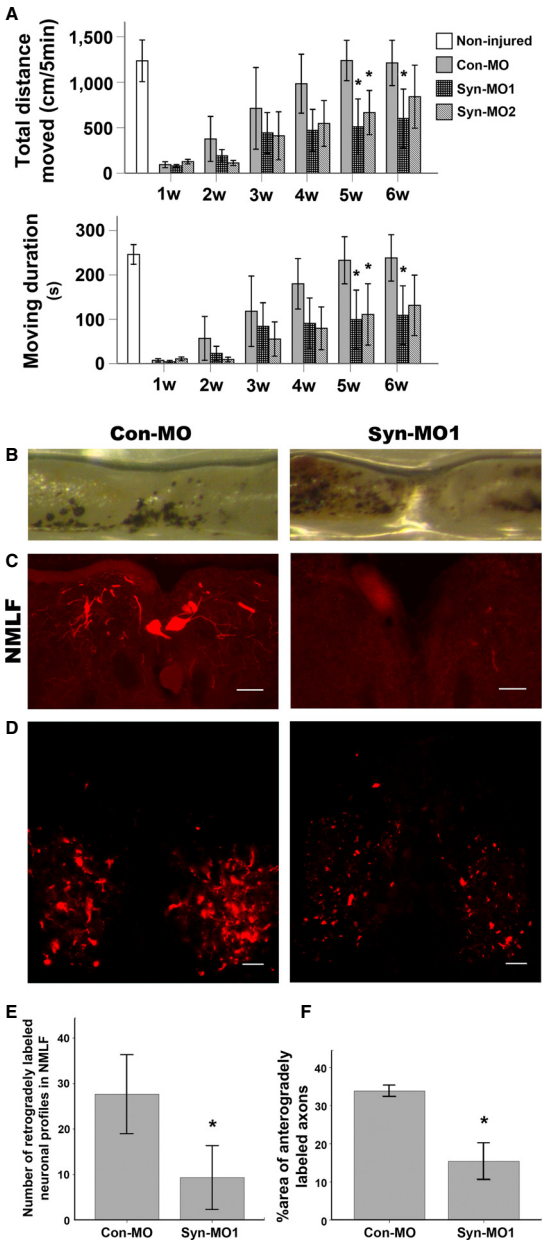

FIG. 6. (A) Locomotor behavior was analysed weekly by swim tracking of fish for 6 weeks (W) post-SCI and treatment with Syn-MO or Con-MO. The total distance moved and duration of movement by fish treated with Syn-MO1 (n = 12 per group) at 5–6 weeks and Syn-MO2 (n = 8 per group) at 5 weeks post-SCI was reduced compared to the Con-MO group (n = 10 per group). *P < 0.05, one-way ANOVA with Tukey's *post hoc* test. (B) At 6 weeks post-SCI, the lesion site had returned to almost the normal size and density in Con-MO-treated fish at 6 weeks post-SCI, in contrast to the overt lesion site of fish treated with Syn-MO1. (C) Retrograde tracing images with biocytin–streptavidin in sections of the NMLF in fish treated with Syn-MO1 at 6 weeks post-SCI. The number of retrogradely labeled neuronal profiles was lower in fish treated with Syn-MO1 than Con-MO. (D) Anterograde tracing images with biocytin–streptavidin in sections caudal to the lesion site 6 weeks post-SCI of fish treated with Syn-MO1 vs. Con-MO. Numbers of axons were reduced in fish treated with Syn-MO1 vs. Con-MO. (E) Quantification of biocytin-labeled neuronal profiles in the NMLF shows that Syn-MO1 treatment reduced the number of retrogradely-labeled neuronal profiles in the NMLF vs. treatment with Con-MO. *P < 0.05, t-test. (F) Quantification of regenerated axonal profiles labeled anterogradely with biocytin indicate that the percentage of labeled area was reduced in cross-sections 0–2 mm caudal to the lesion site of fish treated with Syn-MO1 vs. Con-MO 6 weeks post-SCI. *P < 0.05, t-test. Scale bars, 50 μm.

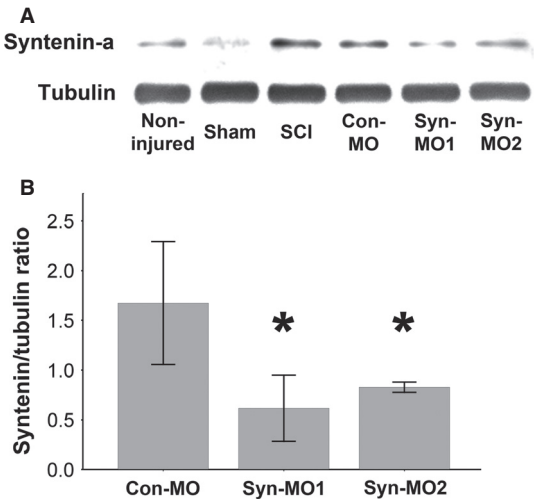

FIG. 7. (A) Western blot analysis of syntenin-a and tubulin in the spinal cord caudal to the lesion site from samples of non-injured fish, spinal cord-injured and sham-injured fish, as well as injured fish treated with Con-MO, Syn-MO1 or Syn-MO2 at 11 days post-SCI. (B) Quantification of syntenin-a/tubulin ratios. Both Syn-MOs reduced syntenin-a expression at 11 days post-SCI. *P < 0.05, one-way ANOVA with Tukey's *post hoc* test.

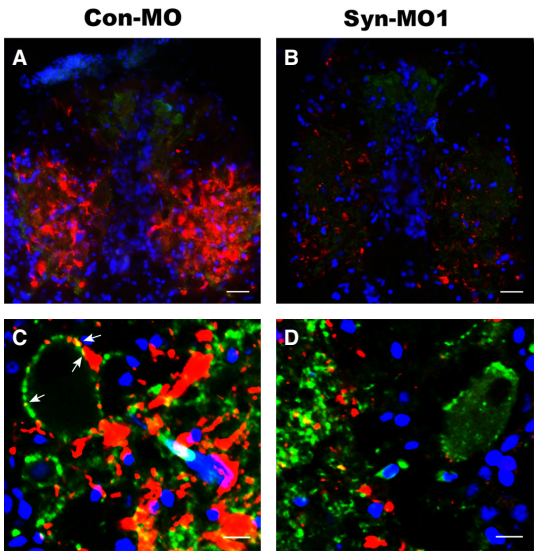

FIG. 8. Double labeling of the anterograde tracer biocytin–streptavidin (red) with the synaptic marker SV2 (green) in cross-sections of the spinal cord caudal to the lesion site 6 weeks post-SCI of fish treated with (A and C) Con-MO or (B and D) Syn-MO1. Newly formed synapses co-labeled with biocytin and SV2 (yellow, arrows) were decreased in fish treated with Syn-MO1 vs. control MO. Blue, DAPI nuclear signal. Scale bars, 20 μm (A and B), 10 μm (C and D). [Color version of figure available online].

although it is not known whether syntenin-a is downregulated in the adult non-injured zebrafish nervous system. Whether regulated expression in the adult central nervous system of mammals would permit regeneration after injury remains to be seen.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. Schematic diagram depicting the main functions of syntenin-1 and its homologs in different species.

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Abbreviations

BrdU, bromodeoxyuridine; Con-MO, standard control MO; ISH, *in situ* hybridisation; MO, morpholino; NMLF, nucleus of the medial longitudinal fascicle; PBS, phosphate-buffered saline, pH 7.3; PFA, paraformaldehyde; qPCR, quantitative real-time polymerase chain reaction; SCI, spinal cord injury; SV2, synaptic vesicle protein 2; Syn-MO, syntenin-a MO.

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