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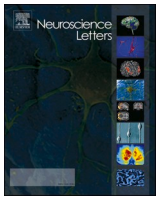

Research article

Asporin and CD109, expressed in the injured neonatal spinal cord, attenuate axonal re-growth *in vitro*

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ABSTRACT

Axonal regeneration is restricted in adults and causes irreversible motor dysfunction following spinal cord injury (SCI). In contrast, neonates have prominent regenerative potential and can restore their neural function. Although the distinct cellular responses in neonates have been studied, how they contribute to neural recovery remains unclear. To assess whether the secreted molecules in neonatal SCI can enhance neural regeneration, we re-analyzed the previously performed single-nucleus RNA-seq (snRNA-seq) and focused on *Asporin* and *Cd109*, the highly expressed genes in the injured neonatal spinal cord. In the present study, we showed that both these molecules were expressed in the injured spinal cords of adults and neonates. We treated the cortical neurons with recombinant Asporin or CD109 to observe their direct effects on neurons *in vitro*. We demonstrated that these molecules enhance neurite outgrowth in neurons. However, these molecules did not enhance re-growth of severed axons. Our results suggest that Asporin and CD109 influence neurites at the lesion site, rather than promoting axon regeneration, to restore neural function in neonates after SCI.

1. Introduction

Spinal cord injury (SCI) can cause irreversible neural disorders in adult mammals due to restricted axonal regeneration across lesions [3,77,90]. Treatment targets the neuronal intrinsic potential and extracellular inhibitory components in axonal regeneration [67]. However, despite progress in understanding the heterogeneous microenvironment of the lesion [22,81], effective treatments to restore neural function are still limited [13]. This suggests that the essential mechanisms underlying neurological recovery are yet to be identified.

In contrast to adults, neonates have significant regenerative potential after SCI, with distinct cellular components, such as microglia [42,82] and neural stem cells (NSCs) [28,74,82], at the injured lesion. In addition to the regenerative potential of neurons [21], the restored spinal cord provides a feasible environment for axonal regeneration in neonates [42]. However, the detailed molecular mechanisms, which are responsible for the enhanced neural restoration in neonates, have not been fully elucidated.

Previously, we reported that distinctive cell types were enriched in adult and neonatal SCI and that neonatal lesions expressed NSC markers [28]. Transplantation of NSCs has been used as a treatment for SCI and has been attributed to neuronal restoration [4,20,31]. In addition to their multipotency to differentiate into neurons and glial cells [65,83], NSCs can release pro-regenerative factors [62], such as neurotrophic factors [24,49] and the extracellular matrix (ECM) [2,53]. Considering that NSCs potentiate the secretion of factors involved in axonal growth,

Abbreviations: AR, Adult reactive cell cluster; ANOVA, Analysis of variance; BSA, Bovine serum albumin; CNS, Central nervous system; DAPI, 4′,6-diamidino-2-phenylindole; d.i.v., Days *in vitro*; d.p.i., Days post injury; DEG, Differentially expressed genes; E17.5, Embryonic day 17.5; EGFR, Epidermal growth factor receptor; ECM, Extracellular matrix; FBS, Fetal bovine serum; GO, Gene ontology; GPI, Glycosylphosphatidylinositol; ICC, Immunocytochemistry; NSCs, Neural stem cells; PFA, paraformaldehyde; PBS, Phosphate buffered saline; PLL, Poly-L-lysine; P1, Postnatal day 1; rhAsporin, Recombinant human Asporin; rhCD109, Recombinant human CD109; rhTGF-β1, Recombinant human TGF-β1; RT, Room temperature; STAT3, Signal transducer and activator of transcription 3; smFISH, Single molecule Fluorescence in situ hybridization; snRNA-seq, Single-nucleus RNA-seq; Mothers againstdecapentaplegic homolog, Smad; SLRPs, Small leucine-rich proteoglycans; TβRII, TGF-β receptor type II; T10, Thoracic 10; TGF-β1, Transforming growth factor β1; 7W, 7-week-old.

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we hypothesized that the predominant molecules released from these cells could promote axonal growth.

Based on detailed analysis of previous snRNA-seq data [28], we focused on Asporin and Cd109, which are highly expressed in neonates with SCI. We found that these molecules were histologically expressed in injured spinal cords of adults and neonates. We also demonstrated that these molecules enhanced neurite outgrowth, but inhibited the re-growth of severed axons *in vitro*. Our results suggest that Asporin and CD109 might restore neural function not through axonal regeneration, but by regulating neurites in the neonatal lesion.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from CLEA Japan. The animals were housed in standardized cages with a 12-hour light–dark cycle and provided with food *ad libitum*. All experimental procedures were approved by the Institutional Animal Care Committee of Osaka University and were carried out in accordance with the Regulations on Animal Experimentation at Osaka University (permission number 29-058-066).

2.2. Spinal cord injury

Seven-week-old (7 W) female mice were intraperitoneally anesthetized (medetomidine 0.3 mg/kg (Orion Pharma); midazolam 4 mg/kg (Astellas); and butorphanol 5 mg/kg (Meiji Seika Pharma)) and underwent thoracic 10 (T10) laminectomy and dorsal hemisection at a depth of 1000 μm with a 27-gauge needle [60]. To sever the central canal of the lesion, the needle was passed at least three times across the spinal cord. Male and female postnatal day 1 (P1) mice were used for neonatal SCI. Neonatal mice were anesthetized with inhaled isoflurane (2 %; Pfizer) and underwent T10 laminectomy and dorsal hemisection at a depth of 500 μm using a 31-gauge needle [28].

2.3. Analysis of snRNAseq and bulk RNA-seq data

We analyzed snRNA-seq and bulk RNA-seq data to characterize the transcriptional profiles of adults and neonates at the lesion sites after SCI [28] (DRA010904). Briefly, this dataset comprised nuclei obtained from injured or sham-treated spinal cord tissue of 7 W adult mice and P1 neonates 7 days post-injury (d.p.i.).

For the snRNA-seq datasets, read alignment and count matrix generation were performed using the Mappa pipeline v1.0 (https://www.takarabio.com/products/automation-systems/icell8-system-and-software/bioinformatics-tools/cogent-ngs-analysis-pipeline) as previously described [28]. Briefly, the reads were mapped to GRCm38 [87], including all introns and exons. We used R v3.5.3 [64] package (R Foundation), Seurat v3.2. [8] to identify marker genes of the clusters using "*Findmarkers*" function with the Wilcoxon rank sum test. Differentially expressed genes (DEG) were selected with a cutoff p-value of 0.0001. For the pathway analysis, gene ontology (GO) terms enrichment analysis of DEG was performed using Metascape v3.0 (http://metascape.org) [91]. For bulk RNA-seq, reads were mapped to GRCm38 [87] and aligned using STAR v2.7.9. (https://github.com/alexdobin/STAR) [17]. The mapped reads were assigned to genomic features using featureCounts v2.0.1 (https://bioinf.wehi.edu. au/featureCounts/) [43].

2.4. Single molecule fluorescence in situ hybridization (smFISH)

smFISH of the injured spinal cord was performed as previously described [28]. Mice were transcardially perfused with ice-cold phosphate buffered saline (PBS) and then 4 % paraformaldehyde (PFA; Nacalai Tesque, 02890–45). The entire spine was collected and post-fixed three nights at 4 °C. Then, we removed the vertebral column and put the spinal cord into 30 % sucrose in phosphate buffer for 48 h at 4 °C. The spinal cords were set in Tissue Tek OCT compound (Sakura Finetek) and stored at −80 °C prior to sectioning. The 14-μm-thick sagittal sections were made with a cryostat and were mounted on MAS-coated glass slides (Matsunami Glass). We used RNAscope probes (Asporin 502051, Cd109 450021; Advanced Cell Diagnostics (ACD)) and stained the sections with an RNAscope Multiplex Fluorescent Kit v2 (ACD, 323100) following the manufacturer's protocol. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Dojindo Laboratories, D212). A confocal laser scanning microscope (FV3000; Olympus) was used to acquire the images.

2.5. Neurite outgrowth assay

Cortical neurons were obtained from C57BL/6 J mice on embryonic day 17.5 (E17.5). In each experiment we used 7 to 9 embryos, and performed 2 independent experiments as technical replicates. The cerebral cortices were dissected and incubated with 2.5 % trypsin (Gibco; Thermo Fisher Scientific) and 50 mg/ml DNAase (Sigma-Aldrich) for 15 min at 37 °C to be dissociated, followed by washing and trituration in DMEM containing 10 % fetal bovine serum (FBS).

Neurons (5×10^4 cells/ml) were suspended in Neurobasal medium (Gibco) containing 2 % B27 supplement (Gibco) and 1 % penicillin–streptomycin (Gibco) and plated on poly-L-lysine (PLL, Sigma)-coated 48 well plates. For outgrowth assays, plated neurons were incubated for 48 h with recombinant proteins: human asporin (rhAsporin, Abcam) or human CD109 (rhCD109; R&D Systems).

2.6. Axonal re-growth assay

Square microfluidic chambers (450 μm microgroove) were purchased from Xona microfluidics. Using sterile forceps, we carefully placed the microfluidic chambers on PLL-coated 35 mm dishes and gently applied pressure to ensure that the devices were attached to the dishes. Cortical neurons were prepared as described in the neurite outgrowth assay section but at higher cellular concentrations of 1×10^7 cells/ml. We then seeded 14 μl of the neuronal suspension on one side of the microgroove and placed in a 37 °C incubator for 20 min to allow the neurons to adhere. All the four wells were filled with 150 μl of medium, and we confirmed that the cells remained in one side. The medium was replaced every 2 days. The axons extended to the other side of the axonal compartment after 5–7 days. Axotomy was performed on day 7 using a vacuum aspirator as previously described [61]. Successful axotomy was confirmed under a microscope (Axiovert 40C; Zeiss). After the axotomy, rhAsporin, rhCD109, and recombinant human transforming growth factor β1 (rhTGF-β1, 10 ng/ml [36]; R&D Systems) or highest amounts of bovine serum albumin (BSA) as control were administered only into the compartment of severed axon, or soma.

2.7. Immunocytochemistry (ICC)

Three days after treatment with the recombinant protein or BSA, the cells were fixed with 4 % PFA. After blocked with 3 % BSA for 1 h at room temperature (RT), cells were incubated with a mouse monoclonal antibody Tuj1 (1:1000; Biolegend) in blocking solution overnight at 4 °C. After three washes with PBS, the cells were incubated with a secondary antibody (Donkey-anti-mouse IgG Alexa 488 1:500, Thermo Fisher Scientific) for 1 h at RT. Nuclei were stained with DAPI.

Images were acquired using a fluorescence microscope (BZ9000; Keyence). For the neurite outgrowth assay, the longest neurite in each Tuj1-positive neuron was traced and measured manually. For the axonal re-growth assay, the lengths of the longest Tuj1-positive axons in each microgroove was manually traced and measured. All images were analyzed using ImageJ (National Institutes of Health, https://imagej.nih.gov/ij) [66].

2.8. Statistical analysis

The values are shown as the mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or Student's *t*-test was used for the comparison of neurites and axon length, with a cutoff P-values of 0.05. Data were analyzed using Graphpad Prism8 (Graphpad Software).

3. Results

3.1. Cd109 and Asporin are highly enriched in neonatal SCI

To screen candidate genes that are specifically expressed in regenerative neonates, we reanalyzed the snRNA-seq dataset, which showed distinct cellular reactions between adults (7 W) and neonates (P1) after SCI [28]. In this snRNA-seq, the analysis was performed on 500-μm-wide pieces of injured (or sham-treated) spinal cord tissue of adults and neonates at 7 d.p.i., the timepoint of the marked cellular response; the PDGFRB^{+} stromal cell-derived fibroblasts increased only in the adult but not in neonatal injured spinal cords. Unbiased clustering identified 18 clusters covering the major known cell types in the spinal cord, including neurons, astrocytes, oligodendrocytes, oligodendrocyte precursor cells, microglia, endothelial cells, and pericytes. Among the 18 clusters, two clusters were mostly enriched in adult SCI (83 % of two clusters in total) and these clusters differentially expressed genes related to the ECM (*Col1a1*$^{+}$, *Postn*$^{+}$), which we previously named adult reactive cell clusters (AR-1 and AR-2). These clusters also express markers of reactive astrocytes [9 86 44] and disease-associated microglia [32 14]. Therefore, we considered AR-1 and AR-2 as mixed cell types that exist in fibrotic scars and border-forming reactive astrocytes [78]. We also named the neonatal SCI-enriched (74 % of the cluster) cellular cluster ependymal-like cells with ependymal cell markers (*Enkur*$^{+}$ and *Dnah6*$^{+}$) [57], which are physiologically expressed by the central canal. In injured neonatal spinal cords, *Enkur*$^{+}$ cells express NSC markers.

In the present study, we focused on two clusters, AR-2 and ependymal-like cell clusters, because the cellular ratio of these clusters was increased by more than four times in the neonatal SCI group compared to the neonatal sham group (Fig. 1a). AR-1 and AR-2 were both enriched in the adult SCI group compared to the adult sham group, and AR-1 was not observed in the neonatal SCI group. GO analysis of AR-1 and AR-2 showed that the marker genes of AR-1 were involved in neuronal death and the inflammatory response, and the marker genes of AR-2 were involved in ECM organization and organ development (Table S1). GO analysis of ependymal-like cells and other clusters showed the upregulation of cilium-related or developmental process-related terms, as previously described [28].

Together with the bulk RNA-seq data, we selected the candidate neural regenerative genes in each cluster following these criteria; 1) the p-value of marker gene expression is less than or equal to 1×10^{15} in order to include up to the top 200 marker genes [26], 2) the expression level in SCI in bulk RNA-seq is more than twice as that of sham in both neonates and adults [10,68] 3) the gene which can work as an extracellular protein, and 4) its recombinant protein is commercially available but is not clarified the role on neurons (Fig. 1b, Table. S2). Finally, we selected two genes: *Asporin* (in AR-2 cells) and *Cd109* (in ependymal-like cells) (Fig. 1b-d).

3.2. Asporin and Cd109 are expressed in the injured spinal cord of neonates and adults

To confirm the expression of Asporin and Cd109 in the injured spinal cord of neonates and adults, we employed the SCI model of neonates (P1) and adults (7 W) and performed histological analysis at 7 d.p.i. using smFISH. In both the neonates and adults, asporin was only slightly expressed in the injured spinal cord (Fig. 2a, b). Cd109 was expressed in the injured spinal cord and central canal of the neonates (Fig. 2c). In adults, Cd109 was slightly expressed in the injured epicenter and central canal (Fig. 2d). The histological expression of Cd109 was consistent with the snRNA-seq dataset because Cd109 was also expressed in AR cells, not only in ependymal-like cells (Fig. 1d).

3.3. Asporin and CD109 elongate neurites in vitro

Asporin is a member of Class I small leucine-rich proteoglycans (SLRPs), which constitute a major non-collagen component of the ECM [29] and are ubiquitously distributed throughout many tissues [25]. Cd109 is a glycosylphosphatidylinositol (GPI)-anchored protein that is expressed in primitive hematopoietic stem cells [73] and glioblastoma stem cells [18]. However, the roles of these two molecules on neurons had remained elucidated. Considering their high expression in neonatal SCI, we hypothesized that these molecules would positively affect neural growth.

To investigate the direct effects of these molecules on neurons, we cultured cortical neurons from mice at E17.5 in 48 wells and treated them with or without rhAsporin or rhCD109 (Fig. 3a). We determined the concentrations of recombinant proteins to include the range of concentrations used for cultured cells in the previous research [38,46,50,63,80,18]. After 48 h, neurons were stained with Tuj1 antibody (Fig. 3b, c), and neurite length was assessed. Neurite outgrowth was enhanced when the neurons were treated with rhAsporin or rhCD109 (Fig. 3d, e). Although we did not evaluate the cellular death or toxicity in each concentration, the number of neurites (cells) was not different among each concentration (Table S3). These results indicate that Asporin and CD109 promote neurite outgrowth in cultured neurons.

3.4. Asporin and CD109 inhibit axonal re-growth in vitro

We next investigated whether Asporin and CD109 could regenerate lesioned axons in the lesion. To investigate the effect of these molecules on severed axons, we cultured cortical neurons in the microfluidic device, severed the extended axons at 7 days *in vitro* (d.i.v.), and simultaneously applied the recombinant protein to act only on the axonal stump (Fig. 4a). Neurons were stained with Tuj1 antibody at 10 d.i.v. (Fig. 4a), and the length of Tuj1-positive re-growing axons was assessed (Fig. 4b, c). The rhAsporin and rhCD109 inhibited axonal re-growth in the dose dependent manner (Fig. 4f, g).

These results indicated that Asporin and CD109 inhibited axonal regrowth if they work on severed axons. Because both Asporin [51] and CD109 [6,7,19] inhibit TGF-β1 signaling, we next assessed the axon regrowth treated with rhTGF-β1 [36]. As expected, rhTGF-β1 enhanced axonal re-growth (Fig. 4d, h). These opposite effects of Asporin/CD109 and TGF-β1 suggest that the attenuated axonal regeneration by Asporin and CD109 is associated with their inhibitory effects on TGF-β1 signaling in neurons.

The distribution of TGF-β receptors in cultured hippocampi neurons from E18 rat are polarized during the development [84]. TGF-β receptors were diffusely distributed in neurons at 2 d.i.v., then they were enriched within the axon compared to dendrites at 4 d.i.v. To prove that TGF-β effects differently on axon and soma, we applied rhTGF-β1 on the soma. We found that the axonal re-growth was enhanced (Fig. 4e, i), which is the opposite result of rhTGF-β applied on the severed axon (Fig. 4d, h). Although we did not evaluate the localization of TGF-β receptors in the neuron after axotomy, these results suggest that the effect of TGF-β or its inhibitor is opposite when they work on axon or soma.

4. Discussions

In this study, we showed that the neonatal SCI-predominant secretory factors Asporin and Cd109 were histologically expressed in the injured spinal cord of both neonates and adults. We also found that Asporin and CD109 enhanced neurite elongation but inhibited the re-

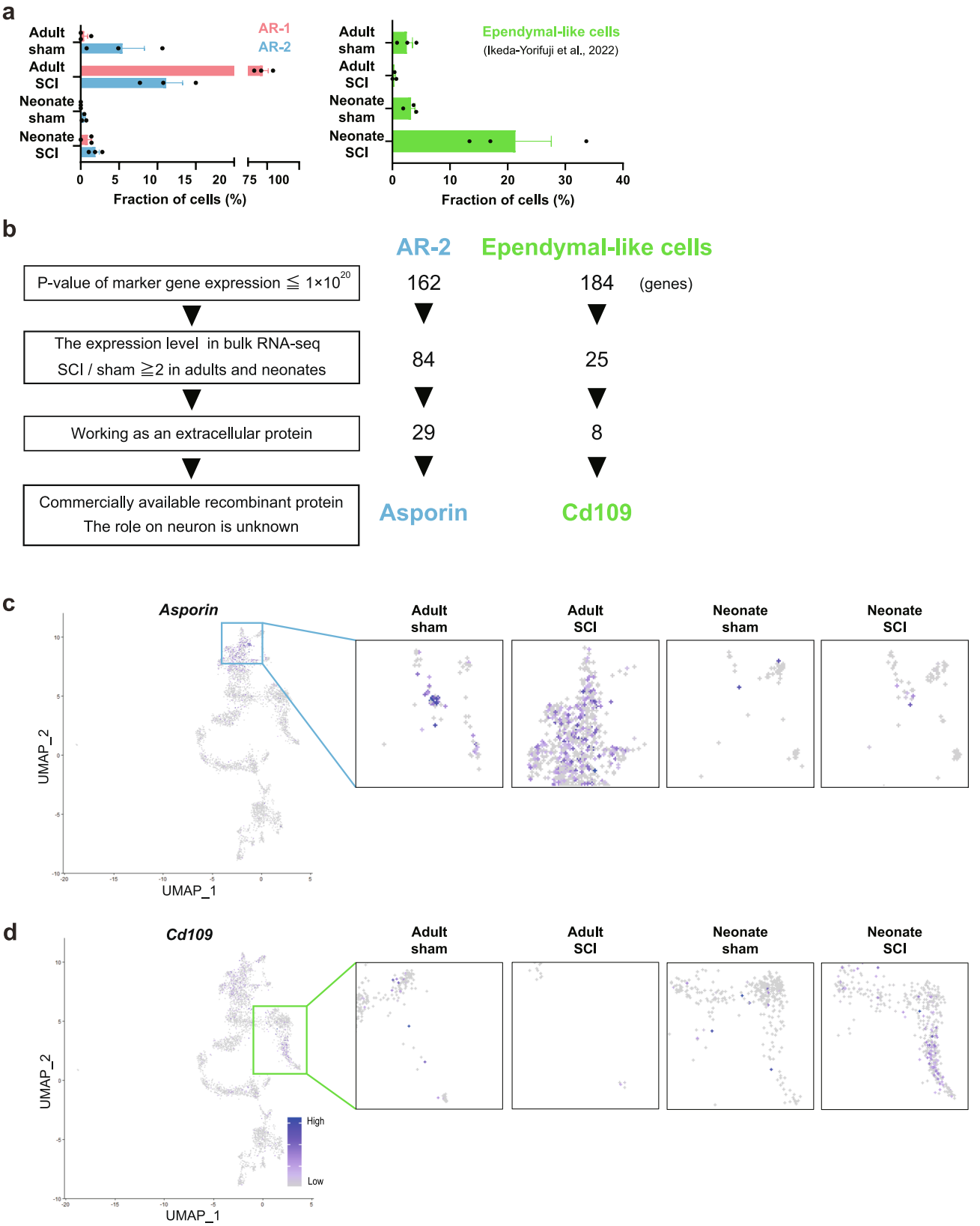

Fig. 1. Asporin and Cd109 are highly expressed in the cells which are enriched in neonates after spinal cord injury. (a) Ratio of the AR (adult reactive cell cluster)-1 and AR-2 cells (left) and ependymal-like cells (right) based on snRNA-seq. The data represent the mean ± s.e.m. (b) The process to narrow down candidate genes for neural regeneration. (c,d) Gene expression of Asporin and Cd109 projected on the UMAP plot. Higher magnification images of AR-2 and ependymal-like cells are indicated by the blue box (c) and green box (d). The color scale indicates the expression values of each gene. Purple dots indicate nuclei with high gene expression.

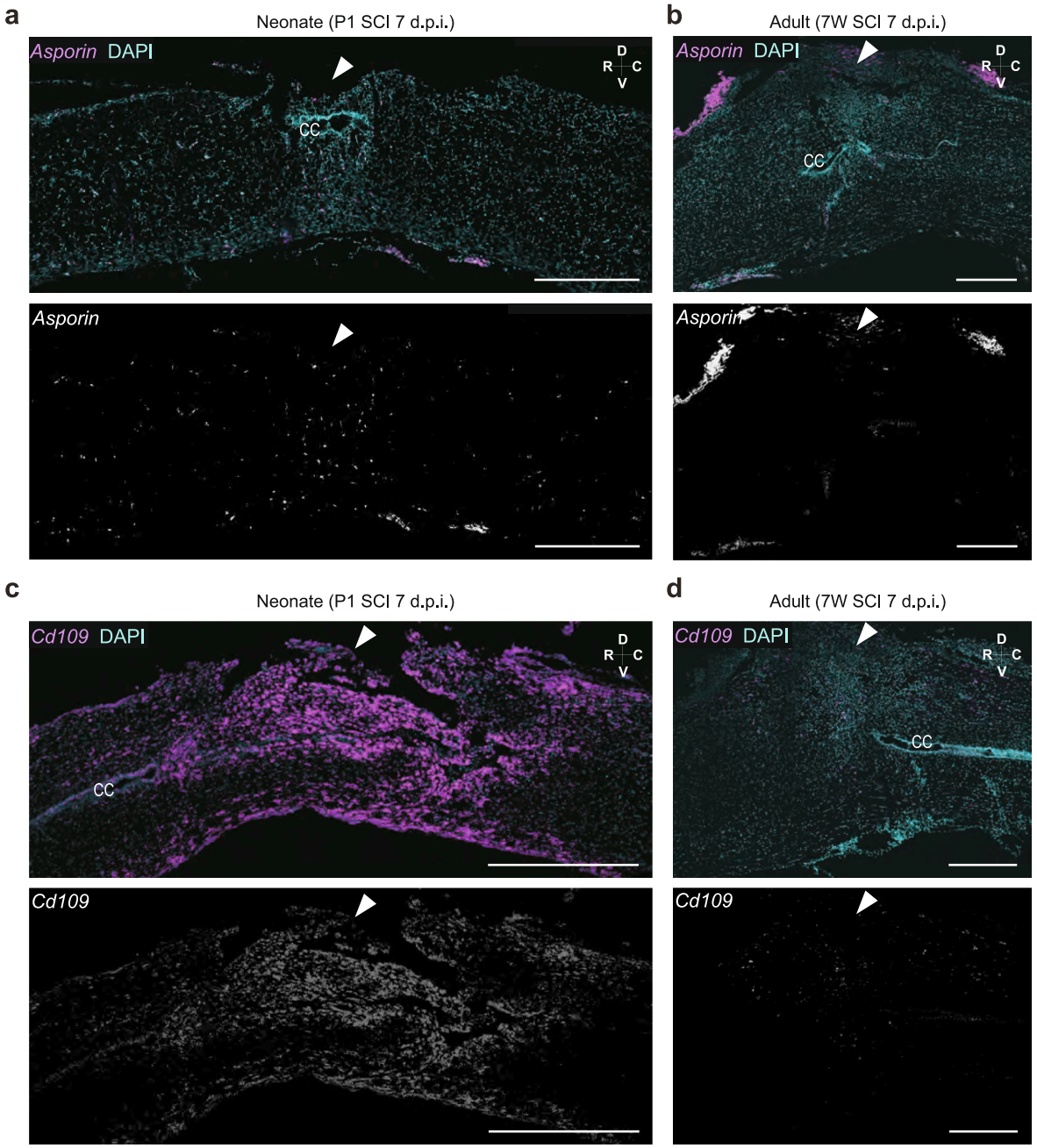

Fig. 2. Histological expression of Asporin and Cd109 in the injured spinal cord of neonates and adults. (a-d) Representative images of sagittal sections of the spinal cords from the neonate SCI (a,c) and adult SCI (b,d) at 7 d.p.i subjected to smFISH (purple) of Asporin (a,b) and Cd109 (c,d) and counterstained with DAPI (nuclei, blue). Scale bars: 500 μm. CC, central canal; C, caudal; D, dorsal; R, rostral; V, ventral. Arrowheads indicate the lesion center.

growth of severed axons *in vitro*.

We first utilized snRNA-seq and bulk RNA-seq data to screen for genes that are enriched in regenerative neonates compared to adults, and finally focused on Asporin and Cd109 as candidates (Fig. 1). In both neonates and adults, Cd109 was expressed in the central canal, supporting that the Cd109 is expressed by "ependymal-like cells" in snRNA-seq (Fig. 2c, d). We also showed that the expression of Asporin and Cd109 was not limited to the injured neonatal injured spinal cord but was also expressed in the injured adult spinal cord (Fig. 2). Although we did not evaluate the cell types that express Asporin or Cd109, these molecules may have different roles in the tissue repair process between adults and neonates, considering the different cellular responses at the lesion center at each age [28,42].

Asporin is expressed in human tissues, such as the osteoarthritic articular cartilage, heart, and liver [48]. Asporin directly binds to type I collagen by the LRR (Leucine-rich repeats) domain and regulates fibrosis in the heart [27] and keloids [47]. Asporin is named based on the presence of a polyaspartate domain and its similarity to decorin [48]. Although the expression of Asporin in the central nervous system (CNS) and its role on the SCI has not been elucidated, Decorin has been well documented in CNS research. Decorin is expressed in neural crest cells, is important for migration in the early embryo [85], and is expressed in neurons and astrocytes. Decorin inhibits the neuronal differentiation of adult NSCs [5]. After CNS injury, Decorin suppresses scarring in the brain [72] and spinal cord [12], thereby lowering scar-derived axonal growth inhibitors [11,12]. Considering the structural similarity between Asporin and Decorin, Asporin might also have an important role in the NSCs niche and injured CNS.

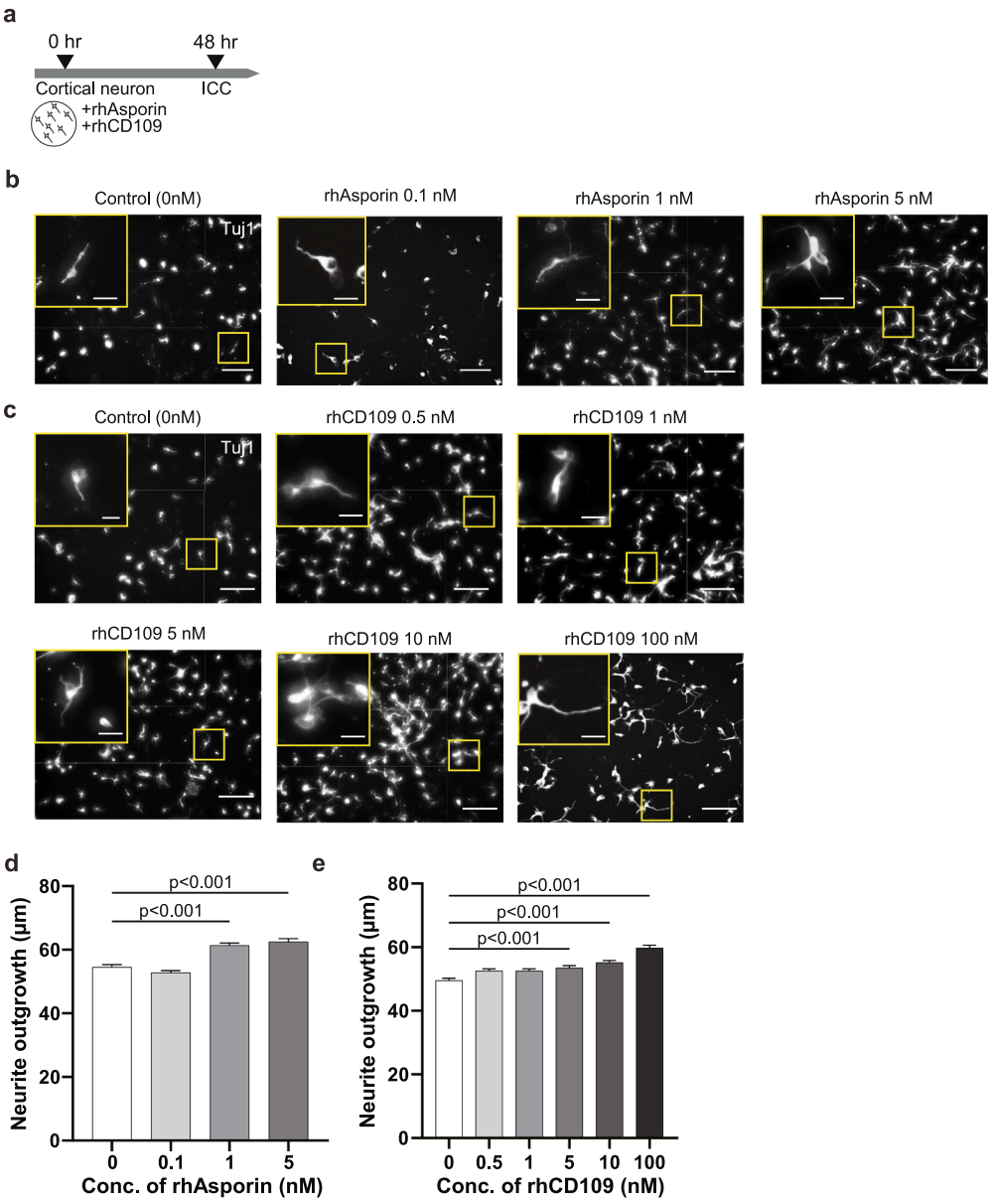

Fig. 3. rhAsporin and rhCd109 elongated neurites *in vitro*. (a) Experimental time scheme of neurite outgrowth assay. (b, c) Representative images of Tuj1+ neurons treated with rhAsporin (b) and rhCD109 (c) in each concentration. Higher magnification images indicated by the yellow boxes are shown on the left. Scale bars: 100 μm, 50 μm in the magnified images. (d, e) The length of Tuj1+ neurites per neuron treated with rhAsporin (d) and rhCD109 (e) in each concentration. One-way ANOVA followed by post-hoc Dunnett's test compared with control. The numbers of axons analyzed in each experiment are shown in Table S3.

CD109 is a GPI-anchored glycoprotein originally found in a subset of hematopoietic stem cells, activated platelets, and T-cells [23,45,70,73]. *CD109* is a gene signature, which defines a subset of reactive astrocytes [44,86] and is also highly expressed in gliomas [18,56,69]. Upregulation of Cd109 has been observed at 7 and 14 days after adult SCI [33], although its role in scar formation and axonal regeneration remains unknown.

Consistent with the snRNA-seq results (Fig. 1c, d), both Asporin and Cd109 were histologically expressed in injured spinal cords of neonates and adults. Asporin was expressed in AR-2; the ratio of this cluster was less than 20 % in each group (Fig. 1a; mean values 0.45, 1.92, 5.44 %, and 11.2 % of the neonate sham, neonate SCI, adult sham, and adult SCI groups, respectively). Reflecting the small population of AR-2 cells, Asporin was only slightly expressed in the lesions of adults and neonates (Fig. 2a, b). We also observed Cd109 expression at the lesion center of adults and neonates (Fig. 2c, d) and in the central canal, which consists of ependymal cells. Considering that *Cd109* is a marker gene of a subset of astrocytes [44,86] and is expressed in ependymal-like cells in snRNA-seq (Fig. 1), the Cd109-positive cells at the lesion might include astrocytes and ependymal cells. For further characterization of these cells, analysis of their colocalization with several cellular markers is warranted in future studies.

Asporin regulates TGF-β, epidermal growth factor receptor (EGFR), and CD44 signaling pathways in cancer cells [88]. Cd109 is a multifunctional coreceptor regulating TGF-β, EGF [54,55,89], and signal transducer and activator of transcription 3 (STAT3) signaling [46]. The regulation of TGF-β signaling by Asporin and Cd109 has been extensively studied; Asporin interacts directly with TGF-β1 binding to its receptor TGF-β receptor type II (TβRII) in cartilage [34,59]. In cancers, Asporin binds directly with TGF-β1 to inhibit its signaling pathway [51], and intracellular Asporin interacts with Smad (mothers against decapentaplegic homolog) 2/3 to activate the TGF-β/Smad2/3 signaling [39]. The primary function of Cd109 is attenuating TGF-β1 signal through working as a coreceptor of TGF-β receptors and increasing TGF-β

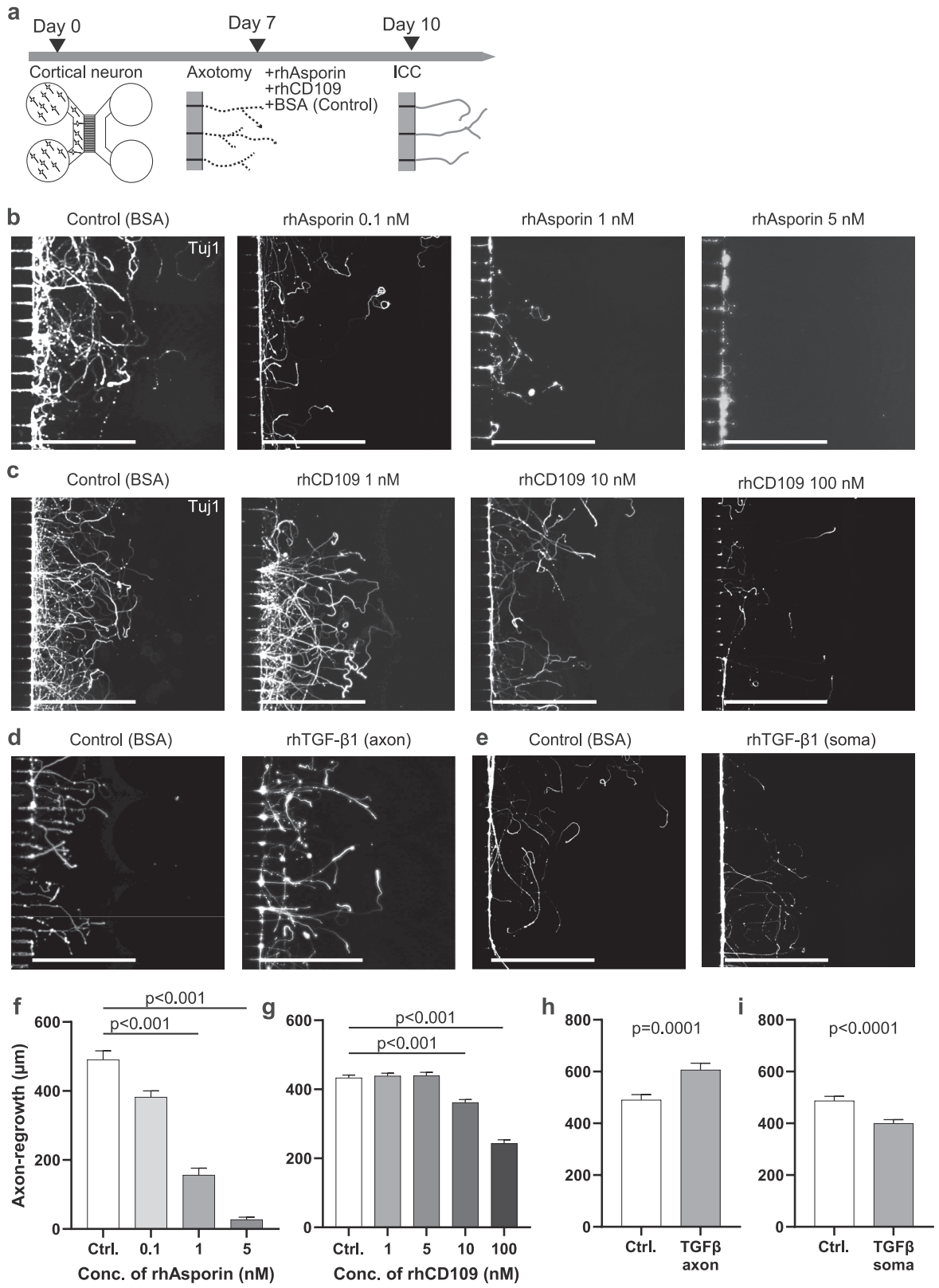

Fig. 4. rhAsporin and rhCd109 inhibited axonal regeneration *in vitro*. (a) Experimental time scheme of axonal re-growth assay. (b-e) Representative images of Tuj1+ regenerated axons treated with rhAsporin (b), rhCD109 (c), rhTGF-β1 on axon sides (d), and rhTGF-β1 on soma sides (e). (f-i) The length of Tuj1+ regenerated axons treated with rhAsporin (f), rhCD109 (g), rhTGF-β1 on axon sides (h), and rhTGF-β1 on soma sides (i). One-way ANOVA followed by post-hoc Dunnett's test compared with control (f, g) and Student's t-test (h, i). The numbers of axons analyzed in each experiment are shown in Table S3.

receptors internalization and degradation [6,7,19]. However, the detailed roles of Asporin and Cd109 in CNS pathogenesis remain unclear.

Previously we reported that TGF-β1 inhibited the neurite outgrowth of cortical neurons *in vitro* [36]. In our present study, we found that TGF-β1 enhanced the axonal re-growth when the recombinant protein is applied to the severed axon (Fig. 4d, h), and inhibited the axonal re-growth when applied to the soma (Fig. 4e, i). The effects of TGF-β on neurite outgrowth depend on the cell type and microenvironment [40,52]. The controversial results have been shown that TGF-β promoted (retinal ganglion cell line [79]) (cortical neuron [41]) or inhibited (cerebellar granule neuron [30,71]) (dorsal root ganglion neuron [16]), which is the outgrowth of the neurons. In the lesioned neuron, TGF-β1 has been shown to promote the axon re-growth (hippocampal neuron [1]) (dopaminergic neuron [35]), corresponding to our results. Considering together, TGF-β1 might have an opposite effect on intact and severed neuron, and this might depend on the polarization of TGF-β receptors on axon or soma [84].

We showed that Asporin and CD109 enhanced the neurite length elongation but not the re-growth of severed axon (Figs. 3, 4); these results are opposite to rhTGF-β1 [36] (Fig. 4h), suggesting that Asporin and CD109 might inhibit TGF-β1 signaling in neurons. In adults, a scar forms on the lesion, which is a major obstacle to axonal regeneration [15,76]. TGF-β upregulates the scar formation [37,58] and the inhibition of TGF-β1 reduces scar formation after adult SCI [36]. Therefore, Asporin and CD109 may decrease scar formation and indirectly enhance axonal regeneration following adult SCI. In neonatal SCI, functional recovery does not completely depend on axonal regeneration across the lesion [75], and partially depends on the adaptation of the spinal circuit. Therefore, it is possible that Asporin and CD109 enhance functional recovery by reconstructing the spinal circuit, despite the inhibition of axonal regeneration from the motor cortex. We previously reported that injury-induced NSCs are enriched in neonatal SCI lesions instead of the scar formation observed in adults [28]. If Asporin and CD109 modify the proliferation and neural differentiation of NSCs, spinal circuit formation may be optimized for functional recovery. Further *in vivo* analysis to elucidate the role of Asporin and CD109 in scar- and injury-induced NSCs is needed to clarify the different cellular reactions between adult and neonatal SCI.

Our study has some important limitations. The receptors for Asporin, CD109, and TGF-β on neurons and their downstream molecular mechanisms were not clarified in this study. Besides the interaction with TGF-β, Asporin and CD109 also regulate several signals as mentioned above. Further analysis of the detailed molecular mechanisms, especially *in vivo* is required to elucidate the potential of Asporin and CD109 as therapeutic targets for neural regeneration.

5. Conclusions

In conclusion, this study revealed that Asporin and Cd109 were expressed in regenerative neonatal SCI. These proteins promote neurite elongation but inhibit axonal re-growth *in vitro*. Our results suggest that Asporin and Cd109 may regulate spinal neurons rather than axonal regeneration at the lesion site, contributing to neonatal neural recovery.

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Author contributions

S. H., I. I., and T. Y. designed the experiments and wrote the manuscript. S.H. and I.I. performed the experiments and analyzed the data. T. Y. coordinated and directed the project. All the authors discussed the results and commented on the manuscript.

CRediT authorship contribution statement

Sakura Hosen: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Iyo Ikeda-Yorifuji:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Toshihide Yamashita:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2024.137832.

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