SCO-spondin from embryonic cerebrospinal fluid is required for neurogenesis during early brain development

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INTRODUCTION

The central nervous system (CNS) develops from the neural tube, a hollow structure filled with embryonic cerebrospinal fluid (eCSF) and surrounded by neuroepithelial cells. Several lines of evidence suggest that the eCSF contains diffusible factors regulating the survival, proliferation, and differentiation of the neuroepithelium, although these factors are only beginning to be uncovered. One possible candidate as eCSF morphogenetic molecule is SCO-spondin, a large glycoprotein whose secretion by the diencephalic roof plate starts at early developmental stages. In vitro, SCO-spondin promotes neuronal survival and differentiation, but its in vivo function still remains to be elucidated. Here we performed in vivo loss of function experiments for SCO-spondin during early brain development by injecting and electroporating a specific shRNA expression vector into the neural tube of chick embryos. We show that SCO-spondin knock down induces an increase in neuroepithelial cells proliferation concomitantly with a decrease in cellular differentiation toward neuronal lineages, leading to hyperplasia in both the diencephalon and the mesencephalon. In addition, SCO-spondin is required for the correct morphogenesis of the posterior commissure and pineal gland. Because SCO-spondin is secreted by the diencephalon, we sought to corroborate the long-range function of this protein in vitro by performing gain and loss of function experiments on mesencephalic explants. We find that culture medium enriched in SCO-spondin causes an increased neurodifferentiation of explanted mesencephalic region. Conversely, inhibitory antibodies against SCO-spondin cause a reduction in neurodifferentiation and an increase of mitosis when such explants are cultured in eCSF. Our results suggest that SCO-spondin is a crucial eCSF diffusible factor regulating the balance between proliferation and differentiation of the brain neuroepithelial cells.

Keywords: SCO-spondin, cerebrospinal fluid, neuroepithelium, neurogenesis, posterior commissure, mesencephalon, subcommissural organ
for its effects are only beginning to be uncovered. One possible candidate as an eCSF morphogenetic molecule is SCO-spondin. This high molecular mass glycoprotein is secreted to the eCSF by the subcommissural organ (SCO), a highly conserved brain gland present throughout the vertebrate phylum (Rodriguez et al., 1992, 1998; Meiniel and Meiniel, 2007). The SCO is one of the first structures to differentiate in the chick brain, expressing SCO-spondin as early as the third day of development (Didier et al., 2007; Caprile et al., 2009). This structure is located at the dorsal diencephalic-mesencephalic boundary, which, according to the prosomeric model, corresponds to the roof plate of prosomere 1, underneath the posterior commissure (PC). The SCO is composed of radial glial cells whose apical domains face the third ventricle and, hence, contact the cerebrospinal fluid, whereas their basal domains extend single processes that cross the nerve bundles of the PC and are attached to the pial membrane (Sterba et al., 1982; Rodriguez et al., 1992, 1998).

In spite of the fact that the sequence of SCO-spondin was reported more than 10 years ago (Didier et al., 2000), its precise function still remains to be elucidated. With respect to its biochemical structure, SCO-spondin is a giant glycoprotein of more than 5000 amino acids that display a multidomain organization, including the presence of several thrombospondin repeats (TSR), low-density lipoprotein receptor type A repeats (LDLRa), EGF-like domains, von Willebrand factor domains (vWF), one emilin (EM1) motif, and a C-terminal cystine knot (CTCK) (Didier et al., 2007). The presence of some of these domains has been reported in other proteins related with neurogenesis like thrombospondin 1 or reelin (Adams and Tucker, 2000; Panteri et al., 2006; Lu and Kipnis, 2010).

SCO-spondin is secreted apically, to the cerebrospinal fluid, as well as basally, toward the extracellular matrix contacting the axons of the PC (Caprile et al., 2009). The best characterized route of SCO-spondin secretion is toward the cerebrospinal fluid where it aggregates and forms the Reissner’s fiber (RF); a thread-like dynamic structure that grows caudally from the SCO through the fourth ventricle and the central canal of the spinal cord, where it is finally degraded at the level of the ampulla caudalis (Molina et al., 2001). The RF has been proposed to regulate CSF production, composition, and circulation (Cifuentes et al., 1994; Rodriguez and Yulis, 2001; Caprile et al., 2003). However, the appearance of RF occurs several days after the onset of SCO-spondin secretion, indicating that, at least during this period, this protein remains soluble in the eCSF. The possible SCO-spondin neurogenic role during early development is suggested by in vitro experiments, where solubilized RF or peptides derived from the SCO-spondin sequence promote the survival (Monnerie et al., 1997) and differentiation (El Bitar et al., 2001) of neuronal cells.

Considering the early secretion of SCO-spondin, its biochemical structure, and the neurodifferentiation effect observed in vitro, we hypothesized that SCO-spondin affects the behavior of neuroepithelial cells during early brain development. To test this hypothesis, we used a loss of function approach in chick embryos by injecting and electroporating a SCO-spondin-specific shRNA expression vector into the neural tube. Our results show that SCO-spondin is crucial for PC formation and for proper brain development. The absence of this protein generates an increase in neuroepithelial cells division in vivo, showing ectopic cellular cluster in the diencephalon and mesencephalon, at the expense of cellular differentiation toward the neuronal lineage. The long-range mode of action of this protein is further supported by in vitro experiments, in which mesencephalic explants cultured in SCO-spondin depleted eCSF leads to a dramatical reduction of neurodifferentiation and an increase in mitosis of neuroepithelial cells.

MATERIALS AND METHODS

CHICK EMBRYOS

Fertilized chick eggs were incubated at 38°C in a humidified incubator for specific time intervals. Embryos were staged according to Hamburger and Hamilton (1992). Experiments were conducted following the guidelines outlined in the Biosafety and Bioethics Manual of the National Commission of Scientific and Technological Research (CONICYT, Chilean Government) and the Ethics Committee of the University of Concepción.

IMMUNOHISTOCHEMISTRY

Embryos were fixed for 24 h in Carnoy, dehydrated in ascending concentrations of alcohols and embedded in paraplast. Brains were oriented to obtain 5–7 μm thick sagittal sections of prosomere 1. Sections were immunostained with mouse monoclonal primary antibodies raised against vimentin and NCAM cytoplasmic domain (H5 and 4D, respectively, from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) as well as with a rabbit anti Reissner’s fiber glycoproteins antibody (AFRU) that recognizes SCO-spondin (Caprile et al., 2009), a mouse anti-βIII tubulin antibody (clone TuJ1, Promega, Madison, WI, USA) and an anti-proliferating cell nuclear antigen (PCNA, PC10 ab29 Abcam). Antibodies were diluted in Tris-HCl buffer containing 1% bovine serum albumin (TRIS-BSA). Goat anti-mouse Alexa-546 and anti-rabbit Alexa-488 antibodies (Invitrogen, Carlsbad, CA) were diluted 1:100 in TRIS-BSA and incubated for 2 h at room temperature. Nuclei were visualized with TOPRO-3 (Invitrogen, Carlsbad, CA). For peroxidase staining, sections were incubated with a secondary goat anti-rabbit IgG coupled to peroxidase (Jackson Immunoresearch, West Grove, PA) diluted 1:100 in the same buffer. Images were acquired with a laser confocal Nikon Eclipse TE2000-U microscope.

The immunohistochemistry of mesencephalic explants was made following the same protocol and using anti-BrdU (G3G4, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti cleaved caspase-3 (ASP175, Cell Signaling Technology), and anti-βIII tubulin (clone TuJ1, Promega, Madison, WI, USA) antibodies.

SCANNING ELECTRON MICROSCOPY

Stage HH18 Chick embryos were fixed for 2 h by immersion in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate. After manually performing a sagittal cut through the midline of the brain, the tissue was dehydrated in ethanol until critical-point drying, ion covered with gold, and examined with an Etec microscope (Etec Corp., Hayward, CA) (del Brio et al., 2000).
**eCSF EXTRACTION**

eCSF from stages HH23–HH34 embryos was obtained as previously described (Gato et al., 2004) with small modifications. In order to avoid contamination with neuroepithelial cells, eCSF was gently sucked up under the dissecting microscope with a glass micro-needle, carefully introduced into the middle of the mesencephalic cavity. To minimize protein degradation, eCSF samples were kept at −15°C with a protease inhibitor cocktail (Sigma P2714), aliquoted, and frozen at −80°C until used.

**WESTERN BLOT**

For immunoblot analysis, 25 μg of total proteins were extracted from stage HH23–34 eCSF or from DMEM (Sigma) conditioned with stage HH36 subcommisural organ explants. Samples were fractionated by electrophoresis in 3%–15% linear gradient sodium dodecyl sulfate polyacrylamide gels and subsequently electrotransferred onto nitrocellulose membrane in a buffer containing 25 mM TRIS-HCl, pH 8.3, 192 mM glycerine, 0.2% SDS and 20% methanol, at 25 mA, for 14 h (Towbin et al., 1979). Non-specific protein binding sites were blocked by incubating the nitrocellulose membranes with 5% non-fat milk in 0.1 M phosphate buffered saline buffer containing 0.1% Tween-20, for 2 h at room temperature. Membranes were probed with the AFRU anti-SCO-spondin primary antibody (1:15,000) overnight. Anti-IgG rabbit secondary antibodies (1:5000) (Jackson Immunoresearch) were incubated for 2 h at room temperature. Immunoreactive proteins were detected with an enhanced chemiluminescence system (SuperSignal, Pierce, Rockford, IL), as instructed by the manufacturer.

**PLASMID CONSTRUCTION**

The shRNA-SCO-spondin plasmid was constructed using the kit siSTRIKETM U6 Hairpin Cloning System- hMGFP (Promega, Madison, WI). The shRNA for SCO-spondin was designed using the programs www.promega.com/sirnadesigner and www.rnaieweb.com/RNAi/siRNADesign. Oligonucleotide sequences were as follows (5′ to 3′): shRNA-SCO-spondin-Forward ACC GGA CAG AGC AGG TAA CAG ATT CAA GAC ATC TGC CCT TTG TCT TC; shRNA-SCO-spondin-Reverse TGC AGA AAA AGG ACA GAG CAG GTA ACA GAT CTC TGT AAT CTG TTA CCT GCT CTG TC; Scrambled-Forward TGC AGA AAA AGG ACA GAG CAG GTA ACA GAT CTC TTG AAT CTG TTA CCT GCT CTG TC; Scrambled-Reverse TGC AGA AAA AGG ACA GAG CAG GTA ACA GAT CTC TGT AAT CTG TTA CCT GCT CTG TC; shRNA-SCO-spondin-Reverse TGC AGA AAA AGG ACA GAG CAG GTA ACA GAT CTC TTG AAT CTG TTA CCT GCT CTG TC; Scrambled-Forward TGC AGA AAA AGG ACA GAG CAG GTA ACA GAT CTC TGT AAT CTG TTA CCT GCT CTG TC.

**INJECTION AND ELECTROPORATION OF shRNA-SCO-SPONDIN in ovo**

The injection and electroporation in ovo was performed as described in Krull (2004) with some modifications. Briefly, the neural tube of HH 9–11 embryos was injected with 1 mg/ml plasmid DNA containing 0.1% Fast Green (Sigma) for visual monitoring of the injection. Several drops of chick Ringer’s solution were dropped onto the embryo after DNA injection. Electrodes were placed above (cathode) and below (anode) the diencephalon. Conditions used for electroporation were five Squarewave electrical pulses of 25 V, 50 ms pulse length, using the Ovodyne electroporator TSS20 (Intracel, Royston Herts, UK) and platinum electrodes. Following manipulation, the eggs were sealed with Parafilm (American National Can™, Greenwich, CT) and returned to the incubator. Twenty-four to thirty-six hours after electroporation, GFP expression was analyzed and embryos displaying expression at the level of the dorsal diencephalon were returned to the incubator until harvesting at HH29.

**ORGANOTYPIC CULTURES OF MESENCEPHALIC NEUROECTODERM**

Organotypic cultures of HH20 optic tecta were performed as described by Gato et al. (2005) and maintained at 37°C with 5% CO2 for 24 h in the presence of 0.01 mM 5-Bromo-2′-deoxyuridine (BrdU, Sigma) and one of the four following media: (1) DMEM (Sigma), (2) SCO-spondin positive conditioned medium from the supernatant of HH36 SCO organ cultures maintained for 4 days in DMEM, (3) 80% DMEM with 20% stage HH25 eCSF, and (4) 80% DMEM with 20% stage HH25 eCSF and supplemented with a 1:300 dilution of the AFRU anti SCO-spondin antibody. After 24 h, the explants were fixed in paraformaldehyde 4% and processed for immunohistochemistry to monitor proliferation (anti-BrdU antibody), apoptosis (anti-caspase 3 antibody), and neuronal differentiation (anti-βIII tubulin antibody). The positive areas of explants stained with each antibody as well as the total explant area were analyzed with the Image J program. Error bars represent s.e.m. and statistical analyses were performed using the Student’s t-test. Differences were considered significant for p < 0.05.

**RESULTS**

**SCO-SPONDIN IS PRESENT IN THE eCSF FROM EARLY STAGES OF DEVELOPMENT**

To precisely describe the spatiotemporal expression pattern of SCO-spondin, we performed immunohistochemical staining on
chick embryo sections from early developmental stages. SCO-spondin was first detected at stage HH17 in the diencephalic roof plate (Figures 1A–D) where it was restricted to the apical domain, suggesting its secretion to the eCSF (arrows in Figure 1D). At this stage, the diencephalic roof plate is similar to the rest of the neuroepithelium, consisting of a pseudostratified epithelium, whose basal and apical domains contact the eCSF and the external limiting membrane, respectively (Figures 1E–F).

The possible secretion of SCO-spondin to the eCSF was confirmed by western blot performed on eCSF from chick embryos at different stages of development (Figure 2). The results show that at HH23 (fourth day of development) the anti SCO-spondin recognizes four bands of 175, 140, 65, and 50 kDa; while at later stages additional bands of 350, 300, and 200 kDa appear, which is in agreement with previous reports (Hoyo-Becerra et al., 2006; Vio et al., 2008). Similar bands are found in the conditioned medium from HH36 SCO explants, with the exception of the smaller bands of 65 and 50 KDa (Figure 2, CM lane).

SCO-SPONDIN BINDS TO THE APICAL DOMAIN OF NEUROEPITHELIAL CELLS in vivo

The presence of SCO-spondin in the eCSF of early chick embryos led us to investigate if this glycoprotein interacts with the apical side of neuroepithelial cells. For this purpose, we realized immunohistochemistry with anti SCO-spondin on sectioned HH23 chick brains embryos (Figure 3). The results show that the immunoreaction is exclusively localized to the cells bodies present in the diencephalic roof plate (Figures 3A,B,D,E). At this stage the immunoreaction covers the entire cell, including the apical region in contact with the eCSF (arrows in Figures 3B,E) as well as the basal region in contact with the NCAM-positive axons of the PC (arrowheads in Figure 3E). The rest of the neuroepithelium is immunonegative for SCO-spondin, except for a thin line covering the apical region of mesencephalic apical membrane (arrows in Figure 3C) and a weak signal observed in the medial and basal part of the mesencephalic cells. In order to confirm the SCO-spondin binding to the neuroepithelial apical membrane, the SCO-spondin antibody was injected to the eCSF of live HH24 embryos. After 24 h, animals were sacrificed and the localization of the SCO-spondin antibody was assessed with anti-rabbit IgG. Our results confirm the binding of anti-SCO-spondin to the apical membrane of neuroepithelial cells in vivo (Figures 4A–C). The negative control, where unrelated antibodies were injected in the same way showed no immunoreaction (Figures 4D–F).

In ovo INHIBITION OF SCO-SPONDIN

In order to analyze the function of SCO-spondin in ovo during early CNS development, we designed a plasmid allowing the co-expression of GFP with a SCO-spondin specific shRNA or with a control scrambled shRNA. The high efficiency of the shRNA was first confirmed on primary culture of chick SCO-cells expressing SCO-spondin, showing that even though the number of transfected cells was low, all of them were immunonegative for SCO-spondin (Figures 5A,B). After the injection of the vector into the neural tube of HH11 embryos (Figure 5C), electroporation of the diencephalic roof plate was performed by placing the positive electrode at the dorsal diencephalic region and the negative electrode beneath the embryo (Figure 5D). One day after electroporation, the GFP expression was monitored in order to ensure that the expression of the plasmid occurred in the accurate region (Figure 5E), and the selected embryos were left to develop until stage HH29 before being examined. From the total of the
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The diencephalon developed normally in animals that received the scrambled shRNA. Such control animals present a SCO-spondin positive region of 700 μm², located precisely below the PC (Figures 6A–C). Under normal conditions, cells of the SCO extend basal prolongations that emerge from the cell body, traverse the PC, dividing this axonal tract in fascicles, and attach to the external limiting membrane (arrows in Figure 6C). The size of the PC fascicles grows progressively in rostro-caudal direction, being smaller at the rostral region. Hence, the length of the basal prolongations of the SCO cells is variable and closely correlates with the thickness of the PC fascicles. At this stage, the primordium of the pineal gland is present at the level of the dorsal diencephalon, rostrally to the SCO (Figure 6A). The diencephalon of animals in which SCO-spondin is inhibited caudally (n = 3) presents a diminution of 32 ± 9% in the PC area, which nevertheless displays a normal degree of axonal fasciculation, and a pineal gland primordium similar to the control (Figures 6D–F). We obtained one animal showing inhibition at the level of the central region, whose phenotype was similar to animals inhibited caudally with a small fasciculated PC, and presence of a normal pineal gland primordium (Figures 6G–I). In such animal, SCO cells have lost their radial morphology and present nuclei in the entire thickness of the SCO. Remarkably, while central inhibition abrogates SCO-spondin expression in the cell bodies, this protein is still strongly detected on the apical side of the SCO cells (asterisk in Figure 6I). Cephalic inhibition of SCO-spondin (Figures 6J–L) does not affect the PC area but causes a higher grade of axonal defasciculation (arrow in Figure 6J). Such animals (n = 3) lack the pineal gland primordium and show ectopic cellular clusters located in the dorsal diencephalon (asterisks in Figure 6J). Likewise, animals with complete inhibition (n = 2, see Figures 6M–O) lack a pineal gland and exhibit ectopic cellular cluster in the dorsal diencephalon (asterisks in Figure 6M). Additionally, complete inhibition causes a drastic diminution in the number of PC axons which are replaced by βIII tubulin positive cell bodies (inset in Figure 6O).

With respect to the mesencephalic region, the optic tectum of WT and control animals that received the scrambled shRNA display a normal and homogeneous thickness of 75±15μm which positively stains for βIII tubulin in its dorsal-most border (Figures 7A–C). The level of cell proliferation was revealed by the presence of a nuclear immunostaining of PCNA in 31 ± 4.3% of the cells (Figures 7D–E). Histological analysis reveals a discrete presence of mitotic spindles on the cells contacting the cerebrospinal fluid (arrows and inset in Figure 7F). The apical border of these cells consists of an homogeneous, uninterrupted, epithelium, and presents immunoreaction for anti SCO-spondin (inset in Figure 7G). Animals in which SCO-spondin was inhibited in the caudal region of the SCO (Figures 7G–L) present a normal optic tectum with a SCO-spondin immunoreactivity at the level of the apical surface (arrows in Figure 7J) and a level of βIII tubulin (Figure 7H–I), and PCNA (Figures 7J–K) immunoreaction similar to the control embryos. By contrast, the general mesencephalic morphology was severely affected in embryos with total inhibition, or in embryos displaying an inhibition localized to the cephalic region of the SCO (Figures 7M–X). Such embryos present a thicker neuroepithelial wall, including the presence of numerous undifferentiated cells (asterisk in Figures 7M,S). βIII tubulin immunoreactivity is highly reduced, particularly in animals with total inhibition (Figure 7U) and the PCNA immunoreactivity is dramatically increased and is present in both the cell nucleus and the cytoplasm. This localization of PCNA has been described in other proliferative cell types, during the M phase of the cell cycle (Iwao et al., 2005). In agreement with these results,
FIGURE 3 | Secreted SCO-spondin diffuses posteriorly and binds to the apical membrane of the mesencephalon. Sagittal sections of dorsal diencephalon and mesencephalon of HH23 chicken embryos. (A–C) Immunohistochemistry with antibodies against SCO-spondin and vimentin counterstained for nuclei with TOPRO-3. (A) Higher magnification of the diencephalic area boxed in (A), showing the localization of SCO-spondin at the level of the apical membrane of the mesencephalic neuroepithelium (arrows in C; (D–E) Immunohistochemistry with antibodies against SCO-spondin and NCAM counterstained for nuclei with TOPRO-3. (E) Higher magnification of the area boxed in (B) showing the localization of SCO-spondin in contact with the axons of the posterior commissure (arrowheads in E) and in the apical region of the SCO cells in contact with the eCSF (arrows in B and E). Di, Diencephalon; Mes, Mesencephalon; PC, Posterior Commissure; PG, Pineal Gland; SCO, subcommissural organ. Scale bars represent 100 μm in (A,C,D); 200 μm in (B,E).

hematoxilin-eosin staining shows a dramatic increase in the number of mitotic cells of mesencephalic neuroepithelium (see arrows in Figures 7R,X, and inset in 7X). Furthermore, the apical border of mesencephalic cells is irregular, with the presence of detached cells (Figures 7R,X), and has lost its immunoreactivity for SCO-spondin (Figures 7Q,U).

In summary, the animals with a SCO-spondin cephalic inhibition have a wider mesencephalon than controls (136 ± 11 μm vs. 75 ± 15 μm, Figure 7A’), and also display a smaller area staining positively for βIII tubulin (9.5 ± 7% vs. 25.6 ± 1.7%, Figure 7B’). By contrast, animals with inhibition of SCO-spondin at the level of the caudal region show a mesencephalon similar to control animals concomitantly with a reduced PC area (67.7 ± 9% compared to control animals, Figure 7C’).

EFFECT OF SCO-SPONDIN ON MESENCEPHALIC EXPLANTS

The mesencephalic malformations found in animals in which SCO-spondin was inhibited led us to investigate the long-range
FIGURE 4 | SCO-spondin is bound to the neuroepithelium apical membranes in vivo. Sagittal sections of dorsal diencephalon and mesencephalon of HH27 chicken embryos. (A–C) Embryos were injected with anti–SCO-spondin antibody and left to develop for 24 h before being sacrificed and immunostained using anti-rabbit IgG. Area boxed in (C) shows the presence of a thin immunoreactive line at the level of the neuroepithelial cells apical membrane. (D–F) Control experiment with an unrelated antibody. Scale bars represent 400 μm in (A,D); 150 μm in (B,E); 200 μm in (C,F).

function of this protein in vitro, using optic tecta explanted from HH20 chick embryos (Figure 8). On the one hand, we analyzed the effect of SCO-spondin gain of function by comparing DMEM with conditioned DMEM medium that has been in contact with SCO explants that secrete SCO-spondin (Figure 2 CM). On the other hand, we performed loss of function experiment by comparing normal eCSF with SCO-spondin-depleted eCSF. The gain of function experiment revealed that conditioned medium from SCO-explants produces a fivefold increase in neurodifferentiation (2.6 ± 1.2 vs. 12.15 ± 1.2; see Figures 8E–F) a threefold decrease in apoptosis (5.92 ± 1.3 vs. 1.6 ± 1.7; see Figures 8A–B) and a diminution in proliferation (6.04 ± 0.9 vs. 3.4 ± 2.6; see Figures 8I–J). Additionally, SCO-spondin inhibition generates a threefold increase in apoptosis (1.2 ± 0.6 vs. 4.8 ± 2.3; see Figures 8C–D) and proliferation (4.4 ± 1.8 vs. 11.4 ± 5.3; see Figures 8K–L), as well as a fourfold decrease in neurodifferentiation (23.4 ± 3.8 vs. 6.2 ± 3.09; see Figures 8G–H). Taken together, these in vitro results are similar to the in vivo situation, where the inhibition of SCO-spondin generates an increment in the mesencephalic proliferation at the expense of neurodifferentiation.

DISCUSSION
In this study, we performed, for the first time, an in vivo inhibition of SCO-spondin expression. Furthermore, by targeting the inhibition to different regions of the diencephalic roof plate, we showed that SCO-spondin is a pleiotropic protein, fulfilling different functions according to its secretion mode (Figure 9). When apically secreted, SCO-spondin remains soluble in the eCSF (Figure 9A) and binds to the apical membrane of neuroepithelial cells, thereby affecting their differentiation and proliferation, while its basal secretion at the level of the PC seems to contribute to the fasciculation and attraction of the PC axons (Figures 9D, E). These results are in agreement with previously reported in vitro experiments in which either SCO-spondin or peptides derived from its sequence promote fasciculation (Stanic et al., 2010), neurite outgrowth (Meiniel et al., 2003; Stanic et al., 2010), and differentiation (Monnerie et al., 1997; El Bitar et al., 2001).

BASALLY SECRETED SCO-SPONDIN REGULATES PC FORMATION
The following previous lines of evidence have led some authors to propose that SCO-spondin contributes to the PC development (Meiniel et al., 2008; Caprile et al., 2009; Hoyo-Becerra...
et al., 2010; Stanic et al., 2010; Grondona et al., 2012): (1) the concomitant formation of SCO and PC, (2) the similarity of SCO-spondin with other molecules involved in axonal guidance, (3) the early secretion of this protein toward the extracellular matrix surrounding the PC axons, and (4) in vitro experiments where the addition of SCO-spondin or peptides derived from its sequence increase neurite length and fasciculation. Here, we provide direct in vivo evidence that SCO-spondin is crucial for PC formation, as its loss of function either causes a marked decrease in the number of axons (animals with total inhibition), a moderate diminution in the number of axons (inhibition at the caudal region), or axonal defasciculation (animals with cephalic inhibition). The different roles observed for SCO-spondin when it is expressed in the cephalic region (fasciculation) and caudal region (incorporation of new axons) could be due to the steep SCO-spondin rostro-caudal expression gradient (Stanic et al., 2010). In this respect it is interesting to note that the presence of integrin β1 (the hypothetical SCO-spondin receptor) in the axonal membrane is negatively correlated to the concentration of its ligand (Condic and Letourneau, 1997). Hence, it is tempting to propose that, in the caudal region, the lower local concentration of SCO-spondin will promote the formation of integrin/SCO-spondin complexes, leading to axonal outgrowth and incorporation of new axons to the PC. According to this model, a higher availability of SCO-spondin in the cephalic region will induce the internalization of surface integrins, diminishing the interaction between the axons and their surrounding extracellular matrix, and, in turn, favoring the interaction between neighboring axons (i.e., fasciculation), a process mediated by axonal adhesion molecules, such as NCAM (Van Vactor, 1998).

**APICALLY SECRETED SCO-SPONDIN REMAINS SOLUBLE IN THE eCSF AND BINDS NEUROEPITHELIAL CELLS**

The apical secretion of SCO-spondin to the CSF and its polymerization to form the RF during late development and adulthood, are widely accepted. However, the presence of a functional and soluble form of SCO-spondin in the eCSF is a matter of recent studies (Hoyo-Becerra et al., 2006; Vio et al., 2008). Our work reveals that from the third day of chick development onward, SCO-spondin is secreted to the eCSF and that it remains soluble at...
least until day 8. We also provide evidence showing that this protein is firmly bound to the apical membrane of neuroepithelial cells at HH24 (fourth day of development), since it is recognized by SCO-spondin antibodies injected to the eCSF in vivo. Our results open new questions regarding the biochemical structure of the soluble form of SCO-spondin detected in the eCSF and to the directionality of its diffusion at this stage. By performing western blots on HH23 eCSF, we have detected the presence of four bands.
FIGURE 7 | Effect of the SCO-spondin loss of function on mesencephalic development. The panels show sagittal sections of mesencephalon of HH29 chick embryos with partial or total inhibition of SCO-spondin. (A–F), Control embryos; (G–L), caudal inhibition; (M–R), cephalic inhibition; (S–X), complete inhibition of SCO-spondin; (A,F,G,L,M,R,S,X), Hematoxilin-Eosin staining; (B,C,H,I,N,O,T,U), Immunohistochemistry for βIII tubulin and SCO-spondin counterstained with TOPRO3. Inset in (C) and arrows in (I) show the apical localization of SCO-spondin. (D,E,J,K,P,Q,V,W) Immunohistochemistry for PCNA counterstained with TOPRO3. Asterisks show ectopic cellular bodies in (M,S,R,X). (A’–C’), Quantification of the phenotypes observed in the SCO-spondin inhibited animals revealing differences in mesencephalic width (A’), percentage of βIII-tubulin positive area with respect to total mesencephalic area (B’), and PC area (C’). Scale bars = 2 mm in (A,B,G,H,M,N,S,T); 50 μm in (C–F,I–L,O–R,U–X).
FIGURE 8 | SCO-spondin regulates the behavior of mesencephalic cells in vitro. Optic tectum explants from HH20 embryos cultured in presence of DMEM (A,E,I); SCO conditioned DMEM (B,F,J); DMEM supplemented with 20% eCSF (C,G,K); DMEM supplemented with 20% eCSF and incubated with anti SCO-spondin antibody (D,H,L). The explants were analyzed for the presence of activated caspase 3 (A′–D′), βIII tubulin (E′–H′) and BrdU incorporation (I′–L′). Panels (A–L) show the merge with the TOPRO3 nuclear signal used to counterstain the tissue. (M–N) Quantification of the area immunopositive for the different antibodies in each experiment. *p < 0.05; **p < 0.01. Bars mean ± SEM.

of 175, 140, 65, and 50 kDa; while at later stages additional bands of 350, 300, and 200 kDa appear. The presence of similar molecular weight bands was found in the CSF of 7 days postnatal rats (Vio et al., 2008) using the same antibody (AFRU) as well as the anti-P15 antibody raised against a peptide derived from the bovine SCO-spondin. These observations suggest the existence of several SCO-spondin isoforms generated by alternative splicing and/or by cleavage. This possibility is in agreement with the presence of several transcripts detected by northern-blot using an SCO-spondin-specific probe (Meiniel et al., 2003).

A smaller 138 kDa human SCO-spondin isoform has been reported (A2VEC9-2, Uniprot), containing eight LDLR-A, two EGF-like and three TSP domains, but lacking the CTCK domain, responsible for oligomerization. Therefore, it remains possible that the 140 kDa SCO-spondin isoform detected in the eCSF at early developmental stages (Figure 2) correspond to this small
understood, since the absence of choroid plexus does not pro-
circulation of eCSF at early stages of development is not yet fully
about how this protein will spread into the brain cavities. The cir-
immunoreactivity.
express SCO-spondin only at the caudal region are devoid of this
cephalic cells. In contrast, the mesencephalic cells of animals that
SCO-spondin immunoreactivity in the apical region of mesen-
cephalic region. Indeed, we found that animals whose SCO-
spondin expression is restricted to the cephalic region display an
cephalic region is sufficient to sustain a normal mesencephalic
dvelopment, while animals with caudal inhibition display an almost
normal morphology.
These results suggest that the region of SCO-spondin inhibi-
tion is more important than the total area of inhibition, since
the presence of few SCO-spondin immunopositive cells in the
cephalic region is sufficient to sustain a normal mesencephalic
development. While it is possible that the SCO-spondin secreted
at the caudal and cephalic region may correspond to distinct
isoforms with different roles on PC and mesencephalic develop-
ment, we favor a second hypothesis according to which the
SCO-spondin secretion pathway differs between the caudal and
cephalic region. Indeed, we found that animals whose SCO-
spondin expression is restricted to the cephalic region display an
SCO-spondin immunoreactivity in the apical region of mesen-
cephalic cells. In contrast, the mesencephalic cells of animals that
express SCO-spondin only at the caudal region are devoid of this
immunoreactivity.
The secretion of SCO-spondin to the eCSF opens the question
about how this protein will spread into the brain cavities. The cir-
culation of eCSF at early stages of development is not yet fully
understood, since the absence of choroid plexus does not pro-
vide the cephalo-caudal directionality of liquid flows observed in
the adult. In this respect, recent studies performed in living
Xenopus laevis embryos report the existence of a semicircular
flow in the telencephalic and mesencephalic cavities, acting the
cerebral aqueduct (the region that contacts the SCO and where
SCO-spondin is secreted) as a bridge between the eCSF of both
cavities (Mogi et al., 2012). The diencephalic roof plate is there-
fore a favored region whose secretions can efficiently spread into
the brain cavities, since they will be carried away both anteriorly
(e.g., toward the pineal gland) and posteriorly (e.g., toward the
mesencephalic cavity).

SCO-SPONDIN AS A MORPHOGEN CARRIER?
One fundamental issue that still remains to be tackled is the
molecular mode of action of SCO-spondin. Our in vitro ex-
periments show that the addition of a SCO-spondin inhibitory
antibody diminishes drastically the ability of native eCSF to pro-
mote neurodifferentiation (Figure 8). It is known, however, that
the eCSF contains a variety of factors involved in brain develop-
ment such as dystroglycan, retinoic acid, FGF2, or LDL (Gato
and Desmond, 2009; Zappaterra and Lehtinen, 2012) suggest-
ing that these factors might influence each other or act redund-
antly. For instance, more than 60% of the neural differentiation
activity exerted by native eCSF requires the presence of LDL
(Parada et al., 2008). Interestingly, to fulfill this role, LDL requires
the presence of others eCSF components that still remain to be
identified (Parada et al., 2008). Considering the presence of
several LDLR-A domains in SCO-spondin, it is possible that SCO-
spondin is involved in the delivery of lipoproteins to neuroepithe-
elial cells. In addition to their function as lipid carriers, LDLR-A
domains can also act as carriers for morphogens of the hedge-
hog (Hh) and Wnt families (Panakova et al., 2005; Willnow et al.,
2007). The association between SCO-spondin and lipoproteins-
morphogens could offer an efficient mean to transport them
around the whole brain cavities, and to increase the local con-
centration of such morphogens. Indeed, if this turned out to be
the case, each morphogens will be presented as multiple copies
on the same lipoprotein particle, generating a multivalent ligand
complex able to promote homomeric clustering of their cognate
receptors, as well as heterodimeric interaction between different
morphogens. Furthermore, the presence of multiple domains in
SCO-spondin like TSP, and EFG-like would increase the range of
combinatorial interactions between extracellular ligands.

In summary, our work strengthens the idea that SCO-spondin
is a multifunctional protein, involved locally in PC development,
and also able to exert a long-range function on remotely located
regions of the brain. The secretion and diffusion of a soluble
form of SCO-spondin into the eCSF allows its binding to the
apical surface of the neuroepithelial cells of the diencephalon
and mesencephalon, where it triggers signaling events promoting
the neuronal differentiation and exit of mitosis. Future chal-
enges will involve deciphering the molecular actors collaborating
with SCO-spondin, such as morphogens, receptors, and signaling
pathways.

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