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Regular Article

Cometin is a novel neurotrophic factor that promotes neurite outgrowth and neuroblast migration in vitro and supports survival of spiral ganglion neurons in vivo

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ABSTRACT

Neurotrophic factors are secreted proteins responsible for migration, growth and survival of neurons during development, and for maintenance and plasticity of adult neurons. Here we present a novel secreted protein named Cometin which together with Meteorin defines a new evolutionary conserved protein family. During early mouse development, Cometin is found exclusively in the floor plate and from E13.5 also in dorsal root ganglions and inner ear but apparently not in the adult nervous system. In vitro, Cometin promotes neurite outgrowth from dorsal root ganglion cells which can be blocked by inhibition of the Janus or MEK kinases. In this assay, additive effects of Cometin and Meteorin are observed indicating separate receptors. Furthermore, Cometin supports migration of neuroblasts from subventricular zone explants to the same extend as stromal cell derived factor 1a. Given the neurotrophic properties in vitro, combined with the restricted inner ear expression during development, we further investigated Cometin in relation to deafness. In neomycin deafened guinea pigs, two weeks intracochlear infusion of recombinant Cometin supports spiral ganglion neuron survival and function. In contrast to the control group receiving artificial perilymph, Cometin treated animals retain normal electrically-evoked brainstem response which is maintained several weeks after treatment cessation. Neuroprotection is also evident from stereological analysis of the spiral ganglion. Altogether, these studies show that Cometin is a potent new neurotrophic factor with therapeutic potential.

Introduction

During development of the nervous system, cell migration, survival and differentiation are supported by neurotrophic factors. These secreted factors are also critical for maintenance and plasticity of the adult nervous system and are furthermore of clinical interest since they may be therapeutic agents for the treatment of neurodegenerative disorders and nerve injuries (Levy et al., 2005). Because of their biologically important and fundamental functions, neurotrophic factors are often well conserved across species and found in families of closely related molecules. Historically, the first neurotrophic factor discovered was "nerve growth factor" (NGF) (Levi-Montalcini, 1987) which is the founding member of the neurotrophin family further consisting of structurally related, brain-derived neurotrophic factor (BDNF)(Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3)(Hohn et al., 1990) and neurotrophin-4/5 (NT 4/5)(Berkemeier et al., 1991; Hallbook et al., 1991). Several such families of related molecules exist where each member often has its own preferred receptor and unique characteristics. Recently, Nishino and co-workers discovered Meteorin as a new neurotrophic factor interestingly not related to any known proteins (Nishino et al., 2004). During mouse development, Meteorin expression

Abbreviations: AP, Artificial perilymph; BDNF, Brain-derived neurotrophic factor; CMTN, Cometin; DCX, Doublecortin; DRG, Dorsal root ganglion; eABR, Electrically-evoked brainstem responses; EST, Expressed sequence tag; FITC, Fluorescein isothiocyanate; GDNF, Glial cell line-derived neurotrophic factor; LC-ESI MS/MS, Liquid chromatography electrospray ionization tandem mass spectrometry; MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight; MS, Mass spectrometry; NGF, Nerve growth factor; NT-3, Neurotrophin-3; NT4/5, Neurotrophin-4/5; SDF1a, Stromal cell derived factor 1a; SVZ, Subventricular zone.

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is largely restricted to the nervous system and in particular to neural progenitors and later, foremost to glial cells. In the adult rodent brain, Meteorin is found in discrete neuronal and glial populations (Jorgensen et al., 2009; Park et al., 2008). Meteorin is a 30 kDa secreted monomeric protein which promotes neurite outgrowth from dorsal root ganglion cultures. In the central nervous system, Meteorin promotes glial differentiation and seems to have an additional role in cerebral angiogenesis. The receptor and exact mechanism of action is unknown but lately Meteorin was shown to use the gp130 co-receptor as an upstream transducer of Jak-STAT3 signaling (Lee et al., 2010). More recently, Meteorin was demonstrated to protect striatal neurons against quinolinic acid induced excitotoxicity in vivo suggesting important neuroprotective properties and a therapeutic potential of the molecule in diseases such as Huntington's disease, stroke, and trauma (Jorgensen et al., 2011). Altogether, Meteorin possesses the properties of a classical neurotrophic factor.

Following the discovery of Meteorin, a similar transcript was automatically annotated as Meteorin-like in public databases but the hypothetical protein and its function remains undescribed. However, using a bioinformatics approach, Ramiliason identified Meteorin-like as part of a large number of transcripts potentially regulated by the subfamily of transcription factors including Pax2, Pax5 and Pax8 (Ramialison et al., 2008). With a specific interest in inner ear development, a smaller number of transcripts were next analyzed by whole mount in situ hybridisation in different developmental stages of medaka fish. Low levels of Meteorin-like mRNA were detected in the otic vesicle and the levels were significantly increased by Pax2/8 overexpression. These data and our interest in Meteorin encouraged us to study Meteorin-like to better understand its biology and explore its therapeutic potential.

To accentuate that Meteorin-like is a distinct and unique molecule we propose the name Cometin (CMTN). In this study we show that Cometin is a well-conserved secreted neurotrophic protein with tissue specific expression during development. In culture, Cometin induces neurite outgrowth from sensory neurons through the Jak-STAT3 and MEK-ERK pathways and furthermore promotes subventricular zone (SVZ) neuroblast migration. In vivo, Cometin has potent neuroprotective properties illustrated by functional and morphological analysis of deafened guinea pigs. Hence, together with Meteorin, the two gene products represent a new family of neurotrophic factors that is structurally unrelated to known protein families.

Materials and methods

Sequence analysis

Homology searches were performed with BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast/). Alignment of amino acid sequences was done using CLUSTAL W (1.7) (Thompson et al., 1994) in the Clone Manager 9 Professional Edition package from Sci Ed Software (Cary, NC). Prediction of signal peptide cleavage sites was done using SignalP (Bendtsen et al., 2004) and prediction of N-Glycosylation sites was done using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Browsers from USCS and Ensembl were used for genomic analysis.

Cloning and production of recombinant mouse Cometin

The coding sequence of Cometin (Meteorin-like from mouse, NM_144797) with a C-terminal histidine tag (GSGSGSHHHHHH) was PCR cloned BamHI/XhoI in pNS1n (Jensen et al., 2002). Correct sequence was confirmed by DNA sequencing (MWG Biotech AG, Germany). FreeStyle™ 293-F cells (Invitrogen, K9000) were transfected with pNS1n-mCMTN-HIS DNA using Lipofectamine 2000 (Life Technologies). The culture was incubated with agitation at 37 °C and 8%CO_2 for three days and separated into cell pellet and supernatant by centrifugation. The supernatant was sterile filtered and recombinant protein purified with TALON Metal Affinity Resin (Clonetech) followed by PD-10 gel filtration (GE Healthcare).

SDS-PAGE and Western blotting

Cell lysates and conditioned media were analyzed by Western blotting as previously described (Fjord-Larsen et al., 2005). Briefly, pNS1n-mCMTN-HIS and mock (pNS1n) transfections were done in parallel and equal amounts of media and lysates loaded respectively to make direct comparison possible. Detection of Cometin was done using custom made monoclonal antibody (mAb#645909) at 0.5 μg/ml and HRP-linked anti-rat Ab (Dako, P0450, 1:2000) as secondary antibody. Alternatively gels were stained with PhastGel™ Blue R (GE Healthcare) according to manufacturer's instructions.

Protein analysis

RP-HPLC, N-terminal sequencing and Mass Spectrometry (MS) was as previously described (Jorgensen et al., 2009). For peptide mass fingerprint analysis and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) de novo peptide sequencing, mCMTN-HIS protein band from SDS-PAGE was reduced, alkylated with iodoacetamide and "in gel" digested with trypsin.

Antibodies

Recombinant mouse Cometin was combined with adjuvant and used to immunize a male Wistar rat. Lymphocytes were collected and fused with X63-Ag8.653 mouse myeloma cells using standard methods (Harlow and Land, 1988). Hybridomas were screened in a direct ELISA for detection of the immunizing protein, and selected wells were subcloned three times resulting in 14 monoclonal antibodies. Furthermore, a female sheep was immunized with recombinant mouse Cometin combined with adjuvant and boosted monthly with antigen. Blood was collected four months after the initial immunization and Cometin-specific antibody purified on an affinity column made from the recombinant Cometin. All antibodies were analyzed by ELISA and western blotting against recombinant Cometin. Furthermore, selected antibodies were screened on rat brains overexpressing Cometin (Supplementary Material, S1). The monoclonal rat antibody mAb#645924 (4 μg/ml) and the polyclonal sheep antibody (0.2 μg/ml) were used for IHC as previously described (Jorgensen et al., 2009).

In situ hubridization (ISH)

Nucleotide sequences corresponding to bp 292–700 of Cometin (NM_144797) and 142–587 of Meteorin (NM_133719) were amplified by PCR with a T7 sequence overhang in the reverse primer. Digoxigenin labeled antisense riboprobes were generated by in vitro transcription using the DIG RNA Labeling Kit from Roche according to the manufacturer. Negative hybridisation controls were similarly generated by PCR with a T7 overhang in the forward primer. In situ hybridisation on sections from E9.5, E10.5, E11.5, E13.5 and E14.5 mice embryos was carried out as previously described (Briscoe et al., 2000). At least three whole embryos were used at each stage.

Dorsal root ganglion (DRG) culture

DRG culture was done as previously described (Jorgensen et al., 2009). Briefly, dorsal root ganglions from around 15 rat P5 pubs (male and female) per set-up were dissociated and plated at a cell density of 1.5×10^4 cells/cm^2. After 1 h, Cometin or Meteorin (R&D Systems) was added at the indicated concentrations in serum-free media. In some experiments, JAK inhibitor I (Calbiochem) and the MEK inhibitor U0126 (Promega) were included at 1 μM. Cells were incubated at 37 °C and 5% CO_2 for 1 day, fixed in 4% PFA and neurons

stained using a β-III-Tubulin antibody (Sigma, T-8660). Digitized images were taken using an Olympus BX61 microscope, and analysis on neurite length per cell was subsequently performed using VisioMorph software (Visiopharm). At least 6–12 images from each of triplicate wells were analyzed for each experimental condition. Experiments were independently performed at least three times.

SVZ Explants

In a typical setup, around eight P2–P3 male rat pups were sacrificed by decapitation and 250 μm brain sections prepared using a vibratome. The SVZ was carefully dissected from the lateral wall of the anterior horn of the lateral ventricle and cut into pieces of 150–200 μm under a dissecting microscope (MZFLIII, Leica). The SVZ explants were mixed with Matrigel (Becton Dickinson) and cultured in four-multiwell plates. After polymerisation, 500 μl Neurobasal medium supplemented with B-27 (Gibco-Invitrogen), N2-factor (Gibco-Invitrgen) and Penicillin/Streptomycin (Gibco-Invitrogen) were added to control cultures. The medium was supplemented with different concentrations of recombinant Cometin or SDF1a (Invitrogen) as indicated. Cultures were maintained in a humidified 37 °C incubator at 5% CO_2. After 24 h, explants were monitored with a DIC microscope and the length of migratory chains from each explant was measured using AxioVision software (Zeiss) and normalized against control. Experiments were independently performed at least three times.

For Doublecortin (DCX) immunocytochemistry, explant cultures were briefly washed with PBS, fixed with 4% PFA in PBS with 0.05% Triton-X 100 and incubated with blocking buffer containing 2% horse serum, 1% BSA, 0.1% gelatin, 0.1% Triton X-100 and 0.05% Tween 20 in PBS. Next, cultures were incubated with anti-DCX (1:100, Santa Cruz) as primary antibodies and Cy3-conjugated anti-goat (1:200, Jackson ImmunoResearch) as secondary antibody. For fluorescein isothiocyanate (FITC)-Phalloidin staining, stimulated and control cultures were rinsed and fixed as described above followed by incubation with FITC-Phalloidin (1:50, Invitrogen). Stained cultures were mounted on glass slides and visualized using fluorescence microscopy (Olympus BX61, Jenoptik C14 camera).

Deafened guinea pigs

All procedures were performed in accordance with the ethical guidelines of Karolinska Institutet, and were consistent with national regulations for the care and use of animals (approval N 35/07). Fourteen pigmented guinea pigs were tested for normal hearing using the Preyer's reflex prior to surgery. All animals received an intracochlear stimulus electrode and an osmotic pump as described previously (Fransson et al., 2010; Maruyama et al., 2008). The stimulus device consisted of a platinum–iridium electrode inserted into the cochlea, and a ground electrode placed in the middle ear cavity. Both electrodes were connected to a percutaneous connector cemented to the skull. At Day 0, the osmotic pump cannula was loaded with ototoxic neomycin to provide intracochlear infusion for 48 h. This caused a hearing loss of 40–60 dB. Animals were then divided in two groups and treated (infusion rate 0.5 μl/h) for 2 weeks with recombinant Cometin (1 μg/ml) or, as a negative control, artificial perilymph (AP) mimicking the inner ear fluid. At the end of treatment, the osmotic pumps were removed but the animals were studied for two additional weeks. The electrically-evoked brainstem responses (eABR) was recorded to assess auditory function at days 2, 7, 14, 21 and 28 as previously described (Fransson et al., 2010; Hall, 1990). Briefly, the animals were anaesthetized and placed in a sound proof box, and eABRs recorded using a SigGen System 2 signal analyzer (Tucker-Davies Technologies). Responses were summed to alternate polarity monophasic current pulses presented at 50 pps. Thresholds were defined as the lowest stimulus level that evoked a reproducible waveform. After the last recording, animals were sacrificed by cardiac perfusion (37 °C saline followed by cold 2.5% glutaraldehyde in 0.1 M phosphate buffer) and the cochleas collected, dehydrated and embedded in Technovit 7100. For stereological analysis, 24-μm thick sections were prepared and stained with hematoxylin and eosin. The optical fractionator (Gundersen et al., 1988) was applied to estimate the total number of spiral ganglion neurons in the Rosenthal's canal using the NEW CAST (v2.14) software from Visiopharm.

Statistical analysis

Data were analyzed by one way Analysis of Variance (one way ANOVA) followed by Multiple Comparisons versus Control Group, Holm–Sidak method. P values are indicated in individual figures.

Results

Cometin is well conserved and forms a unique two-membered family with Meteorin

In public databases, several mouse and human expressed sequence tags (ESTs) are similar to Meteorin and therefore automatically annotated as Meteorin-like. The name Meteorin originally refers to the ability of the molecule to transform glial cells into cells with an elongated tail, looking like meteors (Nishino et al., 2004). To indicate the relationship to Meteorin but also to emphasize the difference between the molecules, we have renamed Meteorin-like to Cometin (after a comet). Translated ESTs are approximately 40% identical to Meteorin and the two molecules form a novel two-membered family of conserved proteins with ten cysteine residues (Fig. 1). Mouse and human Cometin are 77% identical 311 amino acid proteins with a predicted signal peptide and apparently no pro-sequence resulting in a 266 amino acid mature protein with a mass of 30 kDa. According to the NCBI database, Cometin is located on mouse chromosome 11qE2 whereas the human version is found on chromosome 17q25.3. Analysis of EST and genome sequences from other organisms suggests orthologues for Cometin in vertebrates including zebrafish and the frog *Xenopus tropicalis*, whereas no detectable orthologues are found in the invertebrates such as the nematode (*Caenorhabditis elegans*) or the fruit fly (*Drosophila melanogaster*) (Supplementary Material, S2).

Cometin is a classically secreted monomeric novel protein

To examine if Cometin is a secreted protein as predicted, we cloned a C-terminally HIS-tagged version of mouse Cometin (mCMTN-HIS) into a mammalian expression vector and transfected HEK293F cells. From Fig. 2A it is evident that Cometin is effectively secreted and the monomer has a molecular weight of approximately 34 kDa which is including the HIS tag. In cell lysates, Cometin is also found in the monomeric form but some likely dimerization and multimers are also evident. To be able to study biochemical and eventually functional properties of Cometin, recombinant protein was produced in HEK293F cells and purified by metal affinity chromatography. From SDS-PAGE analysis of the purified protein in Fig. 2B, there is only one ~34 kDa band representing recombinant mCMTN-HIS and importantly no visible impurities. The purified recombinant protein is recognized by a monoclonal antibody against Cometin (Fig. 2C) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis suggests that the secreted protein occurs as a monomer of 34 kDa (Fig. 2D). N-terminal sequence analysis of mCMTN-HIS for 10 cycles of Edman degradation did not result in any signals indicating that its N-terminus is blocked. SignalP prediction suggests an N-terminal sequence of QYSSDL… for mature mCMTN (Fig. 1). Cyclization of the N-terminal glutamine to a pyroglutamic acid would result in a negative result in Edman degradation. In order to confirm the N-terminal of purified mCMTN-HIS, it was subjected to “in gel” trypsin cleavage followed by MALDI-TOF peptide mass fingerprinting as well as LC-ESI MS/MS peptide de novo sequencing. The analysis showed that

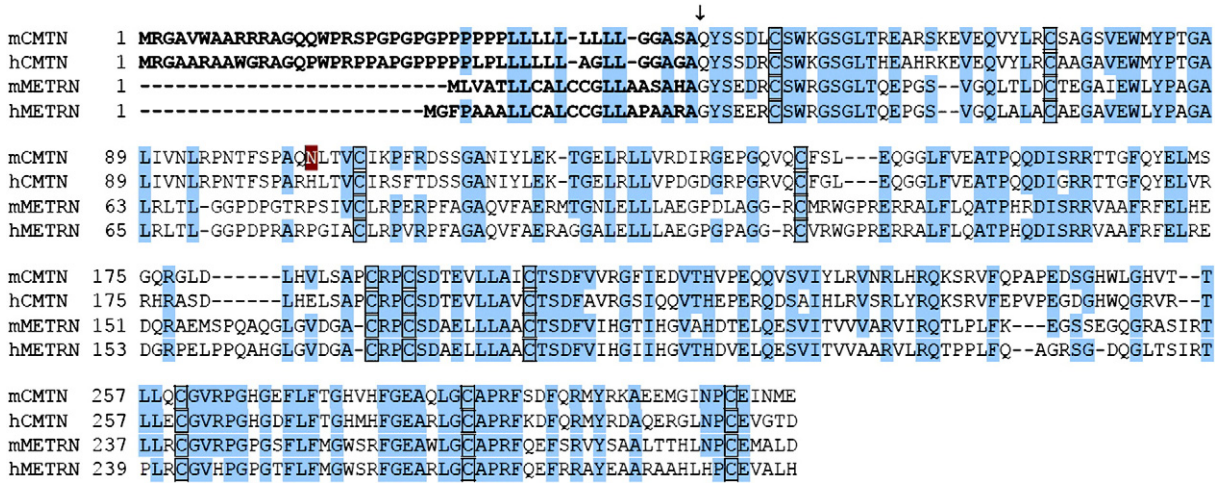

Fig. 1. Cometin is a novel secreted protein forming a family with Meteorin. Amino acid sequence alignment of Cometin (CMTN) and Meteorin (METRN) from mouse and human (m/h). Predicted signal peptide is shown in bold and the cleavage site marked by an arrow (↓). Identical amino acids are shaded and cysteine residues in the mature sequence are boxed. A potential N-glycosylation site in mouse Cometin is marked on a black background.

the digest indeed contained the mass of the suggested tryptic peptide with the sequence of the blocked N-terminal peptide (<QYSSDLCSWK, 1255.544 Da). The analyses also confirmed most of the remaining amino acid sequence of mCMTN-HIS. The calculated molecular mass of mCMTN-HIS with the pyroglutamic acid at the N-terminus, the C-terminal HIS-tag and five disulfide bridges is 31,128.2 Da. However, MALDI-TOF MS resulted in two non-separated peaks with centers corresponding to masses about 33,400 Da and 33,800 Da (Fig. 2D). No signals could be detected at the calculated mass of the polypeptide chain alone, suggesting that the protein is post-translationally modified. Since mouse Cometin has one potential N-glycosylation site (Fig. 1), purified recombinant protein was incubated with N-Glycanase which decreased the molecular weight to ~31 kDa indicating that mCMTN-HIS is indeed a glycoprotein with N-linked oligosaccharides when expressed in HEK293F cells (Fig. 2E). However, the N-glycosylation site in the mouse sequence is not a conserved feature in other species and may therefore be without functional importance (Supplementary Material, S2).

Cometin expression is highly restricted through nervous system development

Initially, Cometin expression was analyzed using semi-quantitative RT-PCR but we did not pick up any significant expression in the more than 30 adult mouse tissues investigated. This is in agreement with the GNF SymAtlas v1.2.4 (Su et al., 2004), where Cometin is not reliably detected in any of the 60 mouse tissues examined by array. This indicates that Cometin is not expressed in adults or, alternatively, restricted to very small cell populations. To address expression at the cellular level, mono- and polyclonal antibodies were generated in rat and sheep respectively by recombinant Cometin immunization. The resulting antibodies were screened by ELISA and immunohistochemistry tested on rat brains manipulated to over express Cometin by unilateral intrastriatal injection of lenti virus (Supplementary Material, S1). The transduced striatal cells thus expressed Cometin at known coordinates with the contralateral non-transduced side functioning as an internal negative control. A few antibodies revealed identical cytoplasmic staining at the injection site while no signal was picked up in the contralateral control side or after omission of primary antibody altogether indicating that the antibodies are specific for Cometin. However, using these antibodies, no significant endogenous Cometin expression was found in coronal sections throughout the adult mouse brain.

To address Cometin expression during development, ISH was done at different embryonic stages (E9–E14.5). The earliest time point at which Cometin could be detected was at E9.5 in floor plate cells throughout the neural tube (Fig. 3A). Between E9.5 and E11.5 Cometin expression is found predominantly in the floor plate cells, but from E13.5, Cometin

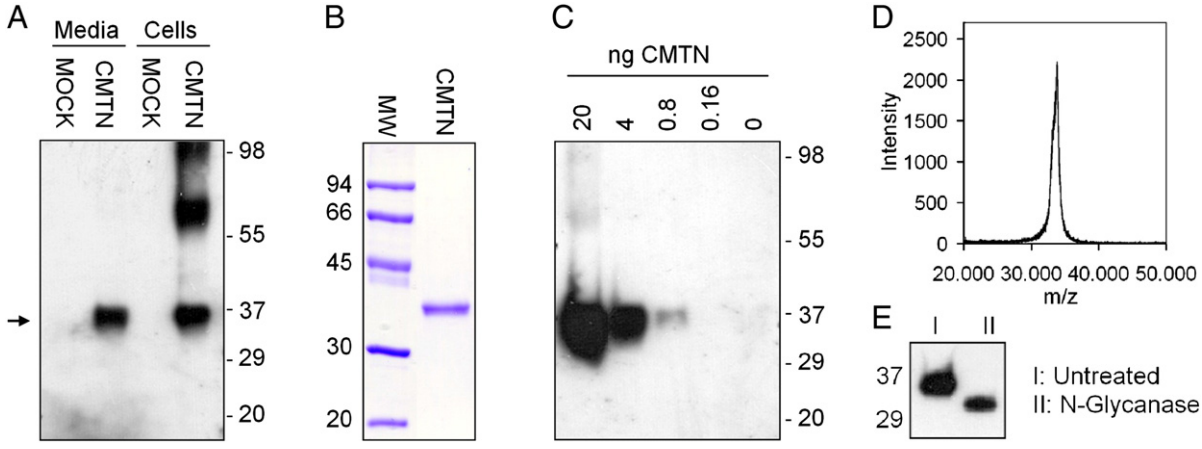

Fig. 2. Characterization of recombinant Cometin. A) Conditioned media and cell lysates from mock and mCMTN-HIS transfected HEK293F cells analyzed by western blotting with an antibody against mouse Cometin. The immunoreactive band corresponding to the mCMTN-HIS monomer is indicated with an arrow (→). B) SDS-PAGE and Coomassie staining of purified recombinant mCMTN-HIS. C) SDS-PAGE and immunostaining of serially diluted purified recombinant Cometin with an antibody against mouse Cometin. D) MALDI-TOF analysis of recombinant mCMTN-HIS showing a mass of 33.8 kDa with a shoulder at 33.4 kDa. E) N-Glycanase treatment of recombinant mCMTN-HIS reduce the molecular weight to ~31 kDa.

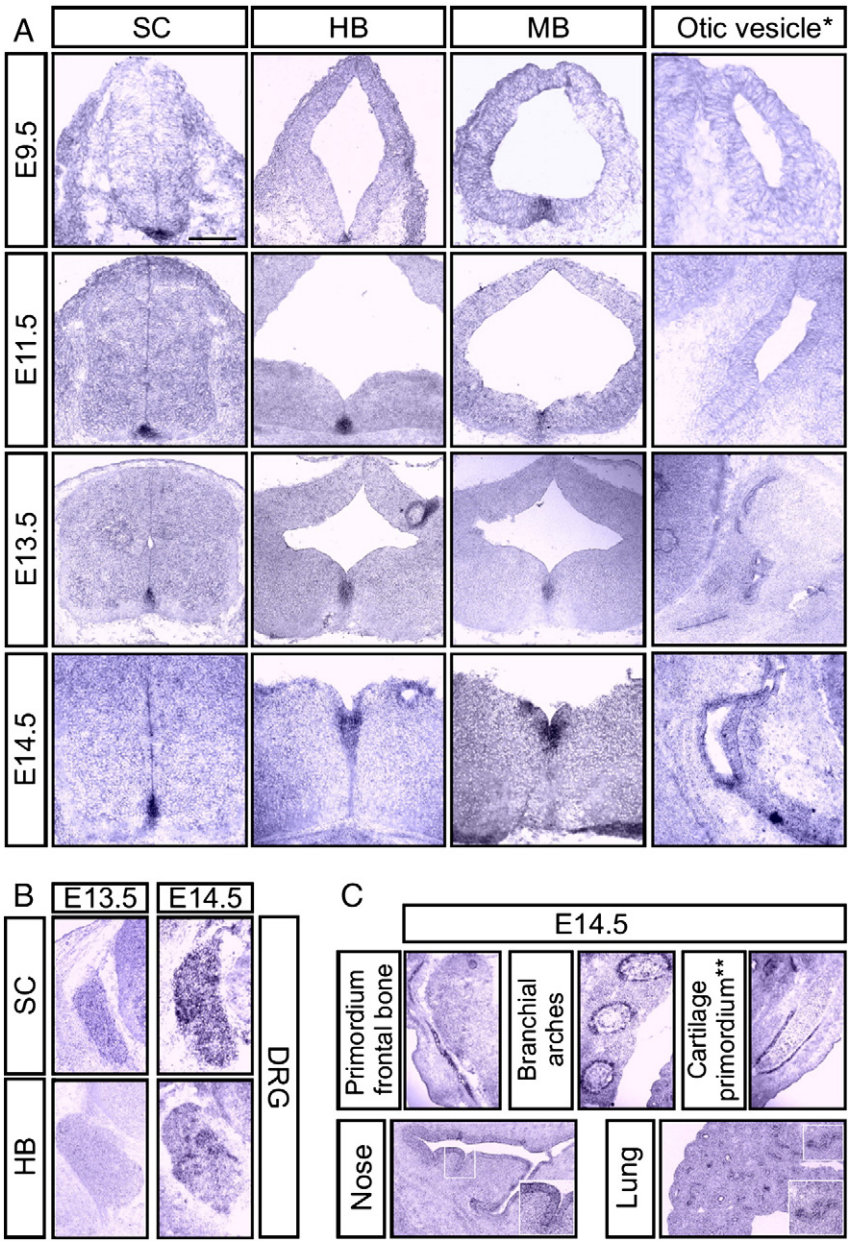

Fig. 3. Cometin expression during development assessed by in situ hybridisation. A) Cometin is selectively expressed in the floor plate throughout the central nervous system from E9.5. From E13.5 Cometin starts to be expressed in the inner ear. B) From E13.5 cometin expression could be detected in the DRGs. C) From E14.5, Cometin is expressed in cartilage primordia, the olfactory epithelia and lungs. * develops into the inner ear. ** the cartilage primordium of the head of the left radius. SC: spinal cord; HB: hind brain; MB: midbrain; DRG: dorsal root ganglia. Scalebar shown in E9.5 SC is 200 μm and applies to all images. See Supplementary Material (S3) for a direct comparison to Meteorin expression.

was also weakly detected in the inner ear with a further increase in expression at E14.5. Similarly, at E13.5, the DRGs begin to express Cometin and a stronger signal was picked up one day later (Fig. 3B). Interestingly, the DRGs located at the hindbrain level seem to have a slightly reduced expression of Cometin compared to DRGs at the spinal cord level. From E14.5, Cometin mRNA was found very strongly expressed in cartilage primordia throughout the embryo exemplified by the frontal bone, branchial arches and radius (Fig. 3C). At this developmental stage, the olfactory epithelium and epithelial layer lining the bronchioles also express some Cometin mRNA. Throughout development, we did not note any differences in the expression pattern between male and female embryos. Together our analysis demonstrates that Cometin expression is developmentally regulated and found only in very few cell populations primarily within the nervous system. A direct comparison to Meteorin expression from E9 to E14.5 can be found in Supplementary Material (S3). It is clear that Meteorin is much more widely expressed during development in agreement with previous expression analysis (Nishino et al., 2004).

To investigate if the Cometin transcript is translated in vivo, we analyzed protein expression at E14.5 through immunohistochemistry (IHC). Generally, the protein expression pattern detected by IHC matches well the mRNA expression pattern detected by ISH (Fig. 4). An intense signal is seen in cartilage primordium, most likely from osteoblasts forming a cell layer over bone surfaces on which matrix is being formed. Furthermore, the floor plate cells express Cometin protein in agreement with the ISH analysis. Low mRNA levels were originally

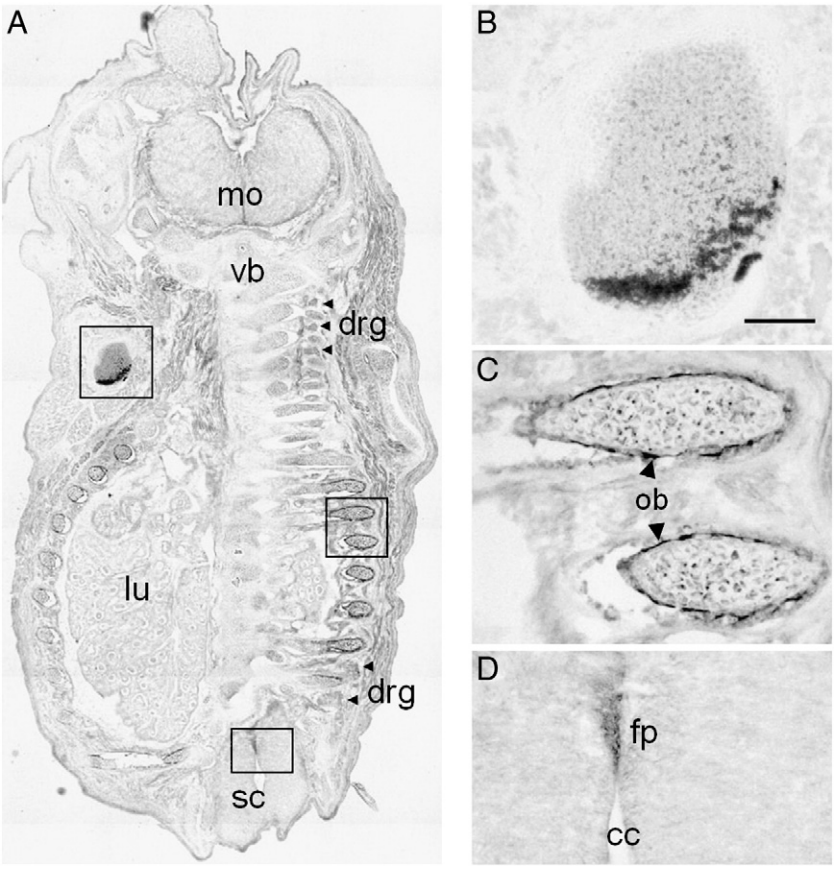

Fig. 4. Cometin expression at E14.5 assessed by immunohistochemistry. A) Coronal section through the caudal level including the medulla oblongata and along the vertebrate to the spinal cord. Note that Cometin expression is restricted to a few discrete locations. B) The mid-shaft region of the right humerus. C) Cometin expressing osteoblasts surrounding the rib surfaces. D) Expression of Cometin in the floor plate. Abbreviations are cc: central canal, drg: dorsal root ganglia, fp: floor plate, lu: lung, mo: medulla oblongata, ob: osteoblasts, sc: spinal cord and vb: vertebrate. Scale bar in B is 100 μm and applies to C and D as well.

detected in lung, DRGs and a few other tissues but this is not evident from the IHC analysis most likely because corresponding protein levels in these tissues were below the detection limit of our antibodies.

Cometin promotes neurite outgrowth from DRGs via the Jak-STAT3 and MEK-ERK pathways

It has previously been demonstrated that Meteorin induce neurite outgrowth from DRGs as effectively as the classical neurotrophic factors NGF, BDNF and NT3 (Nishino et al., 2004). Because the new family member Cometin is likewise expressed in DRGs during development, we tested both proteins on cultures of dissociated DRGs with Meteorin also functioning as a positive control. Without trophic support, neurite outgrowth from DRG cells is very limited whereas in the presence of Cometin, long β-III-Tubulin positive neurites are formed (Figs. 5A and C). The observed neurite outgrowth is dose dependent and not significantly different from that induced by Meteorin. Meteorin is known to function through the Jak-STAT3 pathway with some additional involvement of the MEK-ERK pathway (Lee et al., 2010). In agreement with this, we observed significant inhibition of Meteorin induced neurite outgrowth in the presence of Jak inhibitor I (JAKiI) and marked reduction was also evident with the MEK inhibitor U0126 (Fig. 5B). For Cometin, addition of either JAKiI or U0126 reduced neurite outgrowth to the negative control level. This indicates that both the Jak-STAT3 and MEK-ERK pathways are involved in transducing the effect of Cometin. Interestingly, simultaneous addition of Cometin and Meteorin had an additive effect with significantly more neurites compared to stimulation with each factor alone at saturating doses (Fig. 5D).

Cometin increases SVZ neuroblast migration

Numerous cell lines and primary cultures were investigated in search for biological effects of Cometin. The SVZ of the lateral ventricle contains neural stem cells which give rise to migrating neuroblasts (Alvarez-Buylla and Garcia-Verdugo, 2002). Addition of Cometin to rat SVZ explants caused a significant increase of cell migration and nearly doubled the migratory distance compared to control (Figs. 6A–C). The magnitude of this effect was similar to that of stromal cell derived factor 1a (SDF1a), which is a known potent regulator of in vivo neuroblast migration from the SVZ following brain injury (Imitola et al., 2004; Robin et al., 2006; Thored et al., 2006). Immunostaining of stimulated SVZ explant cultures with antibodies against DCX supported that the migrating cells were indeed neuroblasts (Fig. 6D). The actin cytoskeleton plays a central role in cell migration and SDF1a is known to induce actin polymerisation in multiple systems as an essential part of the migratory process (Jones et al., 2006; Voermans et al., 2001). Using FITC conjugated phalloidin, which specifically binds F-actin, we found that Cometin induces actin polymerisation similar to SDF1a in the SVZ explants as further evidence for an effect on migration (Figs. 6E–G). Taken together, our data demonstrate that Cometin promotes neuroblast migration.

Cometin supports spiral ganglion neuron survival and electrical responsiveness in vivo

We observed developmentally regulated Cometin expression in the inner ear starting at E13.5 (Fig. 3A). Expression of neurotrophic factors are important during inner ear development and furthermore for maintenance in the adult state (Pettingill et al., 2007). To further

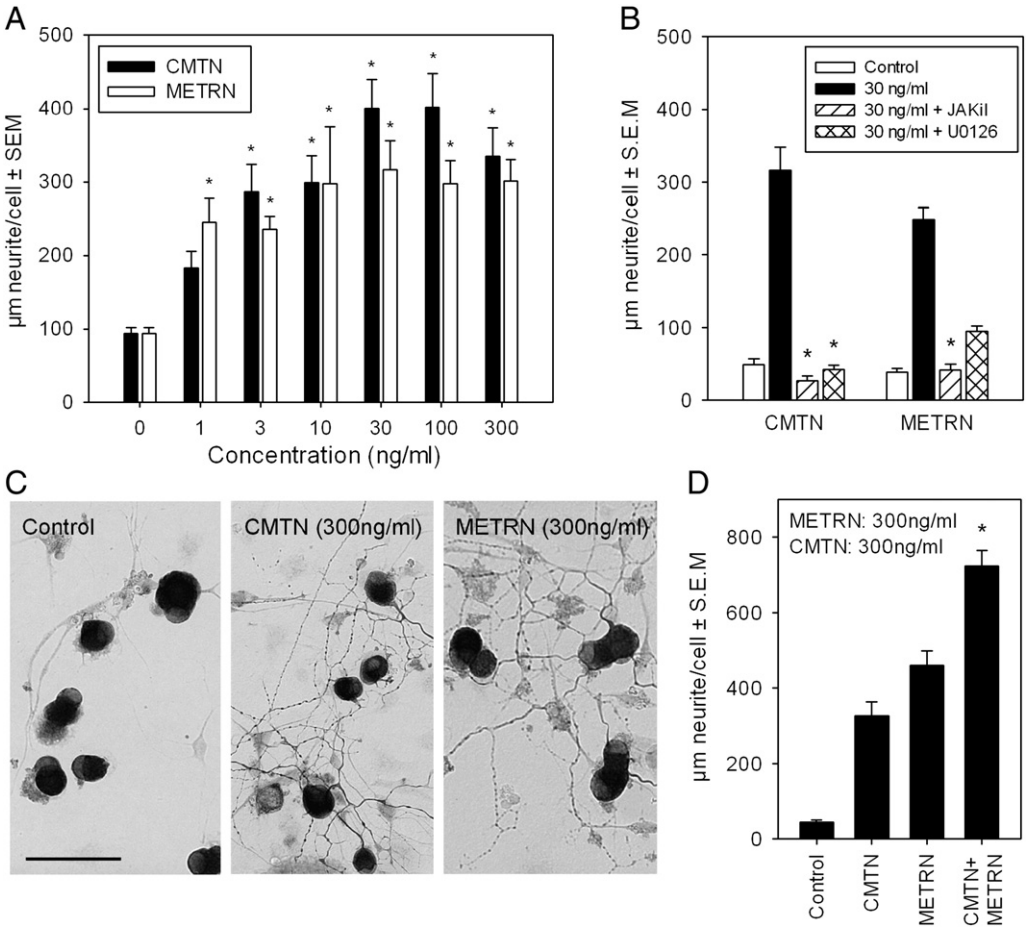

Fig. 5. Cometin induces neurite outgrowth in DRG cultures through the Jak and MEK kinases. A) Quantification of neurite outgrowth showing a similar dose–response relationship for both Cometin and Meteorin. B) Neurite outgrowth induced by Cometin is inhibited by 1 µM JakiI or 1 µM MEK inhibitor (U0126) as is also the case for Meteorin. C) Example of neurite outgrowth from Control, Cometin and Meteorin (300 ng/ml) treated cells. Scale bar: 100 µm. D) Additive effect of Cometin and Meteorin (300 ng/ml). The * indicates a significant difference from addition of either factor alone ($P<0.05$).

investigate the possible role of Cometin in the adult inner ear, we used a guinea pig model allowing both functional and structural studies (Shinohara et al., 2002). In this model, the auditory sensory cells (hair cells) are destroyed by local delivery of the ototoxic agent neomycin to the inner ear. The loss of the sensory cells causes not only permanent hearing loss, but is also accompanied by a secondary degeneration of the auditory primary afferent neurons, the spiral ganglion cells. The progressive degeneration of spiral ganglion neurons can be followed by recording eABR, which give an indication of the electrical responsiveness of the auditory neurons. In the present experiment, a two-day infusion of neomycin produced a significant hearing loss of 40–60 dB. The hearing impaired animals were subsequently divided into two groups and treated by infusion of recombinant Cometin or a saline solution (artificial inner ear perilymph) as a negative control (Fig. 7A). As apparent in Fig. 7B, both treatment groups started out at approximately the same threshold level (~40 µA) but after 2 weeks there was a significant difference ($p<0.001$) between the Cometin treated group retaining normal function and the control group displaying progressive impairment. The pumps were then removed in both groups (day 14). In the control animals, the threshold continued to increase and at the end of the experiment, the eABR threshold was approximately 200 µA. In contrast, the Cometin treatment resulted in a threshold lower than 50 µA throughout the experiment, signifying a positive effect of the treatment. Interestingly, this effect was maintained for at least 2 weeks beyond the treatment period. The protective properties of Cometin were further supported by stereological analysis of the spiral ganglion neurons in Rosenthal's canal. In the Cometin treated group there were significantly ($p<0.001$) more spiral ganglion neurons as compared to the control group (Fig. 7C). Approximately 14,000 spiral ganglion neurons remained in the treated animals compared to only 7000 in the control group. For comparison, the total number of spiral ganglion neurons in a normal guinea pig is approximately 35,000 (Watanabe et al., 2010). The remaining neurons in the Cometin treated group retained normal morphology as evident in Fig. 7D while the few remaining neurons in the non-treated group were irregular and clearly degenerated (Fig. 7E).

Discussion

In this study we show that Cometin is a novel, well-conserved secreted protein that together with Meteorin forms a unique family of neurotrophic factors. This protein family appears restricted to vertebrates, which is also the case for the glial cell line-derived neurotrophic factor (GDNF) family ligands and receptors (Airaksinen et al., 2006) and the neutrophins and their receptors (Lanave et al., 2007). It has been hypothesized that these important families were formed as a result of gene duplication at the origin of the vertebrates as they are required to develop and support a more complex nervous system, which may also be the case for Cometin and Meteorin.

We were unable to detect Cometin in the adult brain using various techniques which is in agreement with public array data (Su et al., 2004; Walker et al., 2004) as well as the Allen Brain Atlas (Lein et al., 2007) and the BGEM in situ database (Magdaleno et al., 2006). Together this suggests Cometin is not expressed in the adult brain. Alternatively, very weak expression or very restricted expression in a

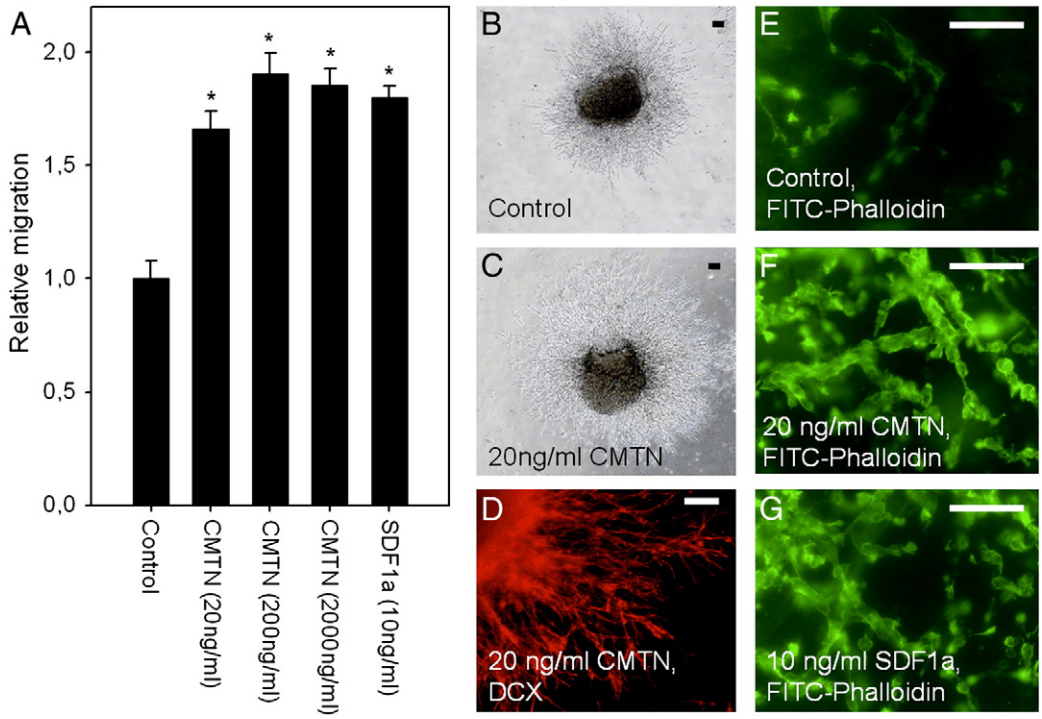

Fig. 6. Cometin stimulates migration of SVZ-derived neuroblasts. A) Quantification of Cometin induced cell migration relative to non-stimulated control (Mean±SEM). SDF1a was included as a positive control. Note that Cometin significantly increase migration (* $P<0.001$). B–C) Light microscopic examples of neuroblast migration increased by Cometin. D) Migrating cells are DCX-positive. E–G) Actin polymerization, visualized by FITC-Phalloidin staining, is induced by Cometin and SDF1a. Scale bars are 50 μm on all images.

small subpopulation may be the case. Very restricted expression is indeed found during development both at the mRNA and the protein level indicating specific rather than general functions of the molecule. Onset of Cometin expression in the inner ear at E13.5 (Fig. 3A) coincides with the appearance of sensory hairs (Ruben, 1967). In medaka fish, the homologous sequence is regulated by PAX transcription factors known to be involved in inner ear development (Ramialison et al., 2008). With the hypothesis that neurotrophic factors expressed in specific regions of the nervous system during development may confer neuroprotection and restoration when reapplied to the same regions during disease in adults, we examined the effect of exogenous delivery of Cometin in adult deafened guinea pigs. Indeed, using both functional and stereological measures, we demonstrate effective Cometin mediated protection of spiral ganglion neurons (Fig. 7). Noticeably, the protective effect was maintained for at least two weeks after treatment cessation, suggesting that the treatment either supported neuronal survival during a critical period or changed the local milieu so that the spiral ganglion neurons could survive despite the loss of trophic support from sensory cells. The effects of Cometin is comparable in magnitude to what has previously been demonstrated using GDNF in the same animal model (Fransson et al., 2010; Maruyama et al., 2008). Deafness is a common disability, which in many cases occurs as a result of loss of sensory hair cell followed by ongoing degeneration of the spiral ganglion neurons similar to the in vivo model used in this study. The functional integrity of the spiral ganglion neurons are important to preserve as the ganglia is target for cochlear implants which circumvents the hair cells and directly stimulate the neurons electrically (Pettingill et al., 2007).

Cometin appears in the DRGs at E13.5 and more intensely at E14.5 (Fig. 3B). During DRG development, the neurons are typically formed around E11.5 whereas the satellite glia cells appear approximately two days later (Lawson and Biscoe, 1979; Sims and Vaughn, 1979). Hence, it is likely that Cometin is expressed by satellite glia surrounding and supporting the neurons in the ganglia. With this assumption and because Meteorin is known to act on DRGs through glia (Jorgensen et al., 2009; Nishino et al., 2004) we used this particular tissue for explant cultures to functionally characterize the two-membered family. We found that Cometin induces neurite outgrowth to the same extent as Meteorin over a wide range of doses but interestingly an additive effect was observed when both factors were added together (Fig. 5). NT3 also works co-operatively with Meteorin in contrast to NGF, BDNF and ciliary neurotrophic factor (CNTF) (Lee et al., 2010; Nishino et al., 2004). Though little is known about the mechanism of action, Meteorin is supposed to have a unique yet unidentified specific receptor through which gp130 is recruited to activate the Jak-STAT3 pathway with a possible downstream involvement of the MEK-ERK pathway (Lee et al., 2010). In agreement with this, we observed more efficient inhibition of Meteorin-induced neurite outgrowth from DRGs with Jak inhibitor I (JAKiI) compared to the MEK inhibitor U0126. For Cometin, we observed complete arrest of neurite outgrowth with both inhibitors indicating that both the Jak-STAT3 and MEK-ERK pathways are involved in the signal transduction. This makes gp130 a likely interaction partner for Cometin but an additional specific receptor is probably required for signaling. Because of the additive effect of Cometin and Meteorin, each ligand would be expected to have a preferred receptor with little cross-reaction. Identification of the specific receptors for Cometin and Meteorin is the next important step in elucidating the mechanism of action employed by this novel protein family.

Cometin mRNA is also found in the floor plate throughout the developing neural tube. This specialized cell population is located on the ventral midline of the neural tube and spans the anteroposterior axis. The floor plate is a well known inductive center that serves as an organizer of ventral cell fate as well as to guide neuronal positioning and differentiation along the dorsoventral axis (Placzek and Briscoe, 2005). The secreted proteins Sonic Hedgehog Homolog and Netrin 1 are key regulators of these respective events. Cometin, being a secreted protein with a similar expression pattern, may well play a role in the differentiation and survival of ventral cell populations generated during embryonic neurogenesis. Throughout the nervous system, newly formed neurons normally migrate away from the proliferative zone towards their final destination. This movement requires signals to start, guide and stop migration as well as changes in the cytoskeleton to mediate

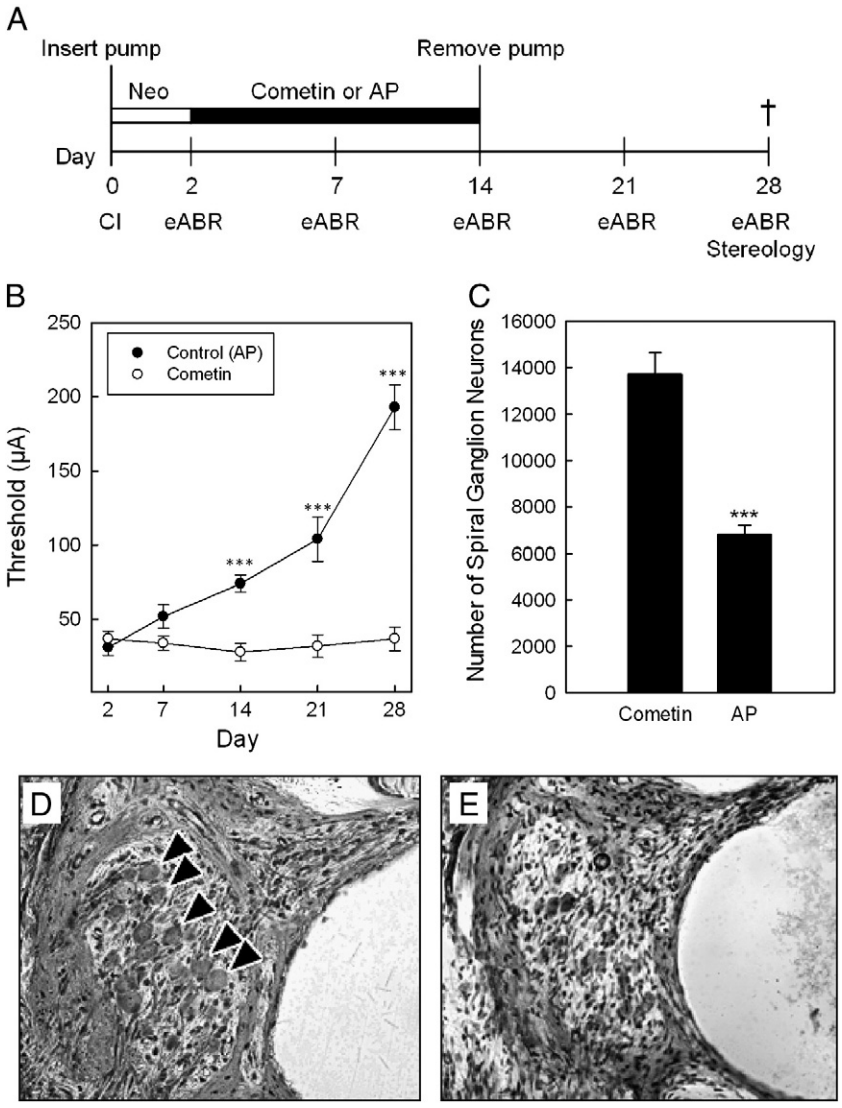

Fig. 7. Cometin protects spiral ganglion neurons in vivo. A) Experimental outline. Briefly, at t = 0, all animals received an intracochlear stimulus electrode connected to an infusion pump. The ototoxic drug Neomycin was infused for two days to deafen the animals. The animals were then divided in two groups receiving Cometin or artificial perilymph (AP) for 12 days after which the pumps were removed. After another two weeks, the animals were sacrificed (†) and the tissue processed for stereological analysis of the inner ear. Electrically-evoked brainstem response (eABR) thresholds were recorded at days 2, 7, 14, 21 and 28. B) Recorded eABR thresholds (Mean ± SEM) throughout the experiment. Note that animals treated with Cometin retained very low eABR thresholds in contrast to AP treated animals. C) Stereological analysis of the total number of spiral ganglion neurons at the end of the experiments (day 28). D and E show the spiral ganglion from animals treated with Cometin and AP respectively. Neurons are indicated by arrowheads in D. *** signifies $p < 0.001$.

the translocation. Using neuroblast explants from the SVZ, we demonstrate that Cometin effectively stimulates migration concomitant with actin polymerisation. Several other neurotrophic/growth factors, e.g. GDNF, BDNF, insulin-like growth factor and vascular endothelial growth factor have previously been reported to be involved in neuroblast migration from the SVZ along the rostral migratory stream towards the olfactory bulb (Sun et al., 2010). Importantly, we find here that the stimulatory action on neuroblast migration by Cometin is comparable to that of SDF1a which is a major regulator of neuroblast migration from the SVZ to the damaged area after stroke (Robin et al., 2006).

Conclusion

This study describes the discovery of Cometin from sequence to biological function in vivo. In summary, Cometin is a well-conserved secreted neurotrophic protein with highly restricted and developmentally regulated expression. It is involved in fundamental processes such as neuroblast migration and neurite outgrowth most likely mediated through the JAK-STAT3 and MEK-ERK pathways. Furthermore, Cometin effectively protects spiral ganglion neurons in vivo and the beneficial effect is maintained after treatment cessation. Also importantly, through these studies, the molecular tools have been created for future investigation of Cometin.

Supplementary materials related to this article can be found online at doi:10.1016/j.expneurol.2011.09.027.

Conflict of interest

JRJ, LFL, MT, TEJ and LUW are employed by NsGene holding patents on Cometin and Meteorin. JPH is employed by R&D Systems selling Cometin and Meteorin reagents for research.

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