In situ hybridization of chemotactically bioactive molecules on cultured chick embryo

Ezequiel J. Tolosa, M. Belén Jaurena, Juan P. Zanin, Natalia L. Battiato, Roberto A. Rovasio

Center for Cellular and Molecular Biology, National University of Cordoba, Argentina

In vitro assays showed that the morphogen, sonic hedgehog (Shh), the chemokine, stromal cell-derived factor-1 (SDF-1), and the growth factor, neurotrophin-3 (NT-3), behaved as chemotactic signals for neural crest cells (NCCs) and are associated with a topological distribution of chemotactic molecules on whole cultured chick embryo. *In situ* hybridization was conducted to show the expression of mRNA transcripts of Shh, SDF-1 and NT-3, while protein immunolocalization and immunolabeling of NCCs were performed on whole mount early chick embryo stages. Spatiotemporal expression of bioactive molecules was shown in the target fields of NCCs of early embryo cephalic sites, supporting the *in vivo* chemotactic behavior of this cell population. This evidence strongly suggests that directional migration of mesencephalic NCCs is modulated by guiding molecules segregated from the optic vesicle region, where NCCs colonize and differentiate into neurons and glia of the ciliary ganglia. The capacity to visualize the *in situ* expression of molecules involved in the chemotactic response of NCCs to concentration gradients established from target regions is intrinsically important for understanding the mechanism of oriented migration of embryonic cells, and also contributes to uncovering additional cell-guiding activities for chemokines, trophic factors and morphogens.

Keywords: Chemotaxis, *In situ* hybridization, Immunolabeling, Neural crest, Whole mount chick embryo

Introduction

During embryo ontogenesis, suitable distribution of cell populations depends on signaling chains which induce a progressive, highly regulated spatiotemporal modulation of developing tissues. Neural crest cells (NCCs) are a paradigm of an embryonic cell population that emigrate from the closing neural tube, disperse toward defined fields, colonize multiple sites with precision and develop many derivatives.[1] It is well known that the efficient migration and distribution of NCCs depends on coordinated genetic and epigenetic factors, triggered by micro-environmental signals.[2–5] However, these are not sufficient to fully explain the modulation of the precise directionality and orientation of NCC dispersion. In recent years, new technologies and knowledge about cell communication at a distance have enabled the re-discovery of the phenomenon of chemotaxis, defined as directional cell migration induced by concentration gradients of diffusible molecules segregated from target sites. This guidance mechanism is present in several prokaryotic and eukaryotic cells.[6–13] Surprisingly, embryo cells, paradigmatic of accurately moving cells, have practically not been dealt with from a 'chemotactic point of view', except in a few systems.[14–17] Studies of directional mechanisms of embryo cells are also important, since many developmental and tumoral anomalies derive from an erroneous distribution/position of these cells.[18–21]

Our recent experimental results have provided *in vitro* evidence of the chemotactic mechanism in NCCs exposed to concentration gradients of embryonic skin extract and stem cell factor, which was proposed as the attractant for subpopulations of melanocyte precursor cells toward its target field, the skin.[22] Moreover, NCCs display chemotactic behavior when exposed to a conditioned medium of optic vesicle cultures, as well as to concentration gradients of the chemokine, stromal cell-derived factor-1 (SDF-1),[23] the trophic factor, neurotrophin-3 (NT-3),[24] and the

Correspondence to: Roberto A. Rovasio, Centro de Biología Celular y Molecular (CEBICEM, FCEFN), Universidad Nacional de Córdoba, Av. Vélez Sarsfield 1611, (5016) Córdoba, Argentina. Email: rrovasio@efn.uncor.edu

One continuing education hour can be earned by reading this article and taking a short test; for details see www.nsh.org

DOI 10.1179/2046023612Y.0000000008

morphogen, sonic hedgehog (Shh).[25] Since it is known that, in early stages of development, a subpopulation of NCCs migrates toward the optic vesicle region to differentiate into neurons and glia of the ciliary ganglia,[1] our hypothesis supports the view that this directional migration is essentially modulated by guiding molecules, such as those mentioned, segregated from the optic vesicle field. Consequently, complementary experiments were necessary to confirm the *in vivo* expression of these bioactive molecules.

In the present work, using whole mount *in situ* hybridization and immunolabeling in cultured chick embryos,[26,27] direct evidence is given of the early expression of mRNA for Shh, SDF-1, and NT-3, as well as the corresponding proteins in the regions where they are expected as a source of chemotactic molecules. This is an essential step toward the validation of the chemotactic hypothesis in whole embryos, through experiments of functional blocking followed by studies on *in vivo* localization and distribution of NCCs.

Materials and Methods

Whole chick embryo culture

The topographic distribution of bioactive molecules and markers was assessed by whole mount *in situ* hybridization and immunocytochemistry on *in vitro* cultured chick embryos derived from fertile *Gallus gallus* Cobb-line chick eggs, pre-incubated for 18–19 hours at 38°C and 80% humidity. This incubation time gives chick embryos at stage HH 4, according to the Hamburger and Hamilton chronological stages describing their external features[28] – conventional nomenclature that will be used throughout this article. The contents of the whole egg were transferred to a bowl in a modified Auerbach's shell-less culture system as described.[19–21,29] Alternatively, the chick embryo blastoderm, excised 10 mm out of the vascular area, was cultured in a wet chamber on a 2.5 cm diameter hemispheric dome of 4% agarose in DMEM medium (Sigma Chem Co., St Louis, MO, USA), resembling the yolk curvature. Cultured embryos were then reincubated in a wet chamber for additional time to complete 45–50 hours (stage HH 12) of embryo development, according to the experimental schedule.

As a culture control, after the initial pre-incubation time, one group of eggs was uninterrupted following the *in ovo* incubation and the chick embryo morphology was double-blind evaluated under a high power stereoscopic microscope, using as a normal pattern the external features described and illustrated in conventional series.[28] No differences were observed between *in ovo* and shell-less cultured groups.

In situ hybridization

Briefly, this method comprises the production of labeled probes, complementary to the target messenger, followed by their presentation to permeabilized cells/tissues under suitable conditions for hybridization to occur. The subsequent development of the probe by means of a specific antibody is followed by a color reaction as a result of phosphatase activity using the corresponding substrates.

Reagents

The following were purchased from Promega (Madison, WI, USA): Wizard Plus Midipreps DNA purification system (Cat. no. A7640); ultra-pure 5′3′ water (Cat. no. W1754); RNasin Plus RNase inhibitor (Cat. no. N2611); and DNase RNase-free RQ1 (Cat. no. M6101). The following were purchased from Roche (Mannheim, Germany): DIG RNA labeling mix (Cat. no. 11277073910); T3 RNA polymerase (Cat. no. 11031163001); SP6 RNA polymerase (Cat. no. 10810274001) and Blocking reagent (Cat. no. 11096176001). All other chemicals were purchased from Sigma Chem. Co. (St Louis, MO, USA), except when another source is stated.

Obtaining and processing chick embryos

Chick embryo cultures incubated to obtain stages HH 4 to 12 were used. The embryos and the surrounding membranes were excised and washed in calcium- and magnesium-free phosphate-buffered saline (PBS), plus 0.1% diethylpyrocarbonate (DEPC), throwing away the vitelline membrane. After fixation with 4% paraformaldehyde in PBS/DEPC at 4°C for 4–12 hours, embryos were washed in PBS/DEPC 3×10 minutes, dehydrated with an increasing methanol series (10 minutes in each: 50, 70, 2×95, $3 \times 100\%$) and maintained in absolute methanol at −20°C until use. Storing the embryos for 5 (or more) days improves the results, because the permeabilizing effect of methanol increases the penetration of reagents and probes.

Preparation of competent bacteria

An isolated colony of *Escherichia coli* culture (DH5alpha lineage) was inoculated to 7 ml of sterile Luria-Bertani medium (LB) [1 g triptone, 0.5 g yeast extract and 1 g ClNa in 100 ml of distilled water, adjusted to pH 6.8–7.2], incubating at 37°C until an optical density (OD_{550}) of 0.3. Five ml of this suspension was added to 100 ml of LB medium and incubated until OD=0.48. After cooling in a water-ice bath for 10 min and centrifuging at 1000*g* at 4°C for 5 minutes, the supernatant was discarded and the

tube inverted on a filter paper to drain the medium. The pellet was resuspended with 20 ml of sterile transformation buffer I (Tfb I) [50 mM $MnCl_2$, 30 mM potassium acetate, 100 mM KCl, 10 mM $CaCl_2$, in 15% glycerol, pH 5.8], and after another step of centrifugation and resuspension with pre-cooled Tfb I, the suspension was left for 5 minutes in a water-ice bath, centrifuged at 1000*g* at 4°C for 5 minutes, and the pellet resuspended with 2 ml of sterile Transformation buffer II (Tfb II) [10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, in 15% glycerol, pH 6.5], and left for 15 minutes in a water-ice bath. Afterward, the suspension was fractionated in 100 µl aliquots and frozen at −70°C.

Transformation of competent bacteria

The vectors used in the transformation were: (1) *pBluescript SK* with a cDNA fragment of chicken Shh of 300 pb (kindly donated by Dr Andrés Carrasco),[30] or with a fragment of 325 pb of chicken NT-3 (kindly donated by Dr F. Hallbook, GenBank access number Z30092).[31] (2) *pDrive* cloning vector with the entire cDNA of chicken SDF-1 of 592 pb (kindly donated by Dr L. R. Read, GenBank access number AY451855).[32]

A thawed aliquot of competent bacteria was transferred to a water-ice bath for 10 minutes and added with DNA plasmid, without exceeding 5% of the volume of competent cells (for 100 µl a maximum of 5 µl of DNA solution was added), and gently mixed. After incubation in a water-ice bath for 30 minutes, the tube was transferred to a water bath at 42°C for 2 minutes without agitation (heat shock). The tube was rapidly transferred to a water-ice bath for 2 minutes, adding 300 µl of LB medium and incubated at 37°C for 45–60 minutes with gentle agitation. A solid LB medium (LB plus 7% agarose) was made and, after cooling and before solidification, the antibiotic was added. In the present work, Ampicillin was used because of the resistance gene of the working plasmid. Aliquots of 50 µl per dish were seeded with a glass comb and incubated for 16–18 hours at 37°C.

Small-scale preparation of plasmid DNA (Quick preps)

This step was applied to verify the quality of the DNA plasmid of transformed bacteria. First, under sterile conditions, a bacteria colony was taken and inoculated to 2 ml of LB medium with the corresponding antibiotic. After incubation with agitation for 16–18 hours at 37°C, the cultures were transferred to microtubes, maintaining the remaining medium in a cool place (bacteria stock). After centrifugation at 1000*g* for 5 minutes, the supernatant was discarded and the tube inverted on filter paper to drain. An aliquot of 100 µl of resuspension solution (RS) [50 mM glucose and 10 mM EDTA in 25 mM Tris-HCl, pH 8.0] was added, mixed by vortex to resuspend and left for 5 minutes at room temperature. Another volume of 200 µl of fresh lysis solution (LS) [0.2M NaOH in 1% (v/v) sodium dodecyl sulphate] was added and mixed by gentle tube inversion without vortexing, and left in a water-ice bath for 5 minutes. After adding 150 µl of cold neutralization solution (NS) [120 ml of 5M potassium acetate and 23 ml acetic acid in 57 ml of distilled water, adjusted to pH 4.8], and mixing by gentle tube inversion without vortexing, the sample was left in the water-ice bath for 5 minutes, centrifuged for 5 minutes at 1000*g* and the supernatant was transferred to another microtube without transferring the sediment (about 400 µl). After adding 2 volumes of cold 100% ethanol (about 800 µl), the sample was mixed by inversion, left 16–18 hours at −20°C (3 hours minimum) and centrifuged at 14 000 *g* for 15–20 minutes at 4°C. The supernatant was removed and the pellet washed carefully with 500 µl of cold 70% ethanol. If the pellet detached, it was centrifuged again. After discarding the ethanol by gentle inversion of the tube, the sample was centrifuged and the ethanol was completely eliminated with a micropipette. Fifty µl of water with 20 µg/ml RNase was added and mixed gently before incubation for 30 minutes at 37°C. To test the presence and quality of DNA, 5 µl of each suspension was mixed on a parafilm sheet with 5 µl of gel-loading buffer (GLB) [0.25% of bromophenol blue or xylene cyanol FF, depending on the probe size, in 30% glycerol], and seeded on agarose gel [500 mg agarose and 0.5 µl ethidium bromide in 50 ml of TBE buffer (45 mM Tris borate and 1 mM EDTA)]. To verify the insert presence or linearize the vector, the appropriate enzyme was used as 5 µl of digested plasmid in a final volume of 25 µl of restriction buffer (RB) [40 µl of commercial buffer 10×, 40 µg DNA, 10 µl of commercial restriction enzyme and milliQ water up to a total volume of 400 µl]; afterwards all the volume was seeded in a gel and electrophoresis was run at 40 mA.

Large-scale preparation of plasmid DNA (Midi preps)

This step allows plasmids to be obtained and recovered with the desired sequence for use as templates of the probes. A volume of 100 ml of 16–18 hours incubation culture was centrifuged at 10 000*g* for 10 minutes at 4°C, then the supernatant was discarded and 3 ml of RS was added to the pelleted cells, avoiding clotting. The mixture was left

on an ice block, 3 ml of LS added, mixed by inversion of the tube, and left for 5 minutes on the ice. After adding 3 ml of NS and mixing by inversion, the tube was centrifuged at 14 000*g* for 15 minutes at 4°C, until a solid pellet formed (otherwise, it was re-centrifuged). The supernatant was transferred into a 15 ml plastic tube without removing the white pellet (or clearing through filter paper) and left on ice.

The DNA purification kit resin was resuspended in a water bath at 37°C for 10–20 minutes, cooled to 30°C and 10 ml of DNA purification resin was added. For each cell culture, a purification column (Midi column) was used, connected to a vacuum pump. The mixture of DNA and resin was transferred to the purification column, which was connected to the vacuum pump. Fifteen ml of the kit washing solution was added and the vacuum pump was started. Another 15 ml of washing solution was added and the vacuum was applied. The purification column was detached by cracking or cutting, and the column content was transferred to a microtube of 1.5 ml and centrifuged at 10 000*g* for 2 minutes to remove any remaining fluid. The column content was transferred to another microtube, 300 µl of nuclease-free water pre-warmed to 65–70°C was added and, after waiting for 1 minute, the plasmid DNA was recovered from the column by centrifugation at 10 000*g* for 20 seconds. The purification column was discarded. The solution recovered was centrifuged at 10 000*g* for 5 minutes, the supernatant was transferred carefully to another microtube, and stored at −20°C.

DIG-Riboprobe synthesis

Obtaining DNA template

The objective of this step is to linearize the plasmid with the corresponding restriction enzyme. ClaI was used for vectors with chShh and chNT-3 sequences, and SphI for the chSDF-1 sequence, which was applied in the RB buffer. Digestion occured for 16–18 hours (3 hours minimum) at the enzyme working temperature. To check digestion, 10 µl of the solution was mixed with 5 µl of BPB buffer, seeded in 1% agarose gel with ethidium bromide and run at 40 mA.

From this step onward, all handling had to be strictly free of RNase. If the digestion was complete, it was extracted with phenol/chisam (1 : 1) 200 µl each, then threefold with 400 µl of chisam, then 40 µl of 3M sodium acetate was added, followed by 800 µl of absolute ethanol. The solution was mixed by inverting the tube. The suspension was maintained at −20°C for 2 hours (or overnight), then centrifuged at 14 000*g* for 20 minutes at 4°C, the pellet was washed with 1 ml of RNase-free 70% ethanol, centrifuged again, the ethanol was discarded and the pellet was dissolved with 25 µl of 5′3′ ultrapure water.

Quantifying the DNA-template

Dilutions between 1/500 and 1/1000 of the linear plasmid were used, completing the volume with autoclaved ultrapure milliQ water or TE buffer [10 mM Tris pH 8.0 and 1 mM EDTA]. The absorbance was measured in a quartz cuvette at 260 and 280 nm wavelength, using the same solvent as used in the DNA dilution as a blank control, and considering that 1 Abs_{260}=50 µg/ml of DNA.[33] The absorbance values at 280 nm enable assessment of the protein contamination of the DNA sample.

Transcription solution

The following transcription mix was prepared at room temperature: 2 µl of 10× enzyme transcription buffer, 2 µl of 10× mixing of DIG-labeled nucleotides (or fluorescent-labeled from Boehringer), 1 µg of linealized DNA, 1 µl of 40 U/µl sinRNA, 2 µl of 20 U/µl RNA polymerase, and 5′3′ water to complete 20 µl. After gentle mixing, the solution was centrifuged and incubated for 2 hours at 37°C. The template was degraded with 1 µl DNase for 15 minutes at 37°C, and the reaction volume was adjusted to 100 µl with TE buffer pH 8.0, containing 0.1% sodium dodecyl sulfate.

Purification of DIG-riboprobe by Sephadex column

The column support was prepared with a tuberculin syringe plugged with glass-wool and oven-sterilized. The syringe was filled with sephadex G-50 (or G-25 coarse) suspension, the end placed into a microtube without lid, and then the entire syringe placed into a 15 ml tube and centrifuged at 800–1000*g* for 2–3 minutes. This procedure was repeated until the sephadex column was 1 cm from the upper end of the syringe. The microtube was replaced by a sterile tube, 100 µl of TE buffer was added, the microtube/syringe was centrifuged for 2–3 minutes and the recovery of a 100 µl volume was verified. The transcription mix was seeded and centrifuged, the solution recovered was transferred to a microtube, 10 µl of RNase free 3M sodium acetate pH 5.2 and 200 µl of absolute ethanol was added, and left for 16–18 hours at −20°C. After centrifuging at 14 000*g* for 20 minutes at 4°C, the pellet was washed with 750 µl of cold 70% ethanol, centrifuged and the remaining ethanol was discarded. The pellet was dissolved with 22 µl of 5′3′ ultrapure water, aliquot of 2 µl was tested.

Checking the probes

One per cent agarose gel without ethidium bromide was fabricated, after all the elements used for

electrophoresis were washed with detergent and immersed for 20 minutes in 3% hydrogen peroxide, then washed with distilled water. Two µl of RNA sample was mixed with 23 µl of the seeding buffer (SB) [230 µl formaldehyde, 26 µl of 1M MOPS pH 7.0], 2.1 µl of 3 M sodium acetate pH 5.2, 1.2 ml of 500 mM EDTA pH 8.0, 0.25 ml of GLB, 0.64 ml formamide and 3 µl of 1 mg/ml ethydium bromide, with a suitable stain, selected according to the probe sizes. Two µl of molecular weight markers of RNA (RNA ladder) were mixed with 23 µl of SB buffer, the samples were heated for 15 minutes at 65°C, cooled for 5 minutes on ice, seeded on the gel and run at 40 mA.

Quantifying the DIG-Riboprobe

Diluted solutions of 1/500 and/or 1/1000 were made, mixing 1 or 2 µl of the transcription solution with water/DEPC or RNase-free TE buffer. The absorbance was then measured using quartz cuvettes at 260 and 280 nm, using the same solvent as used with the sample as a blank control, taking into account that 1 Abs_{260}=40 µg/ml of RNA.[33] The absorbance values at 280 nm enabled assessment of the protein contamination, or purity, of the DNA sample.

In situ hybridization procedure on cultured chick embryo

First day (hybridization)

The embryos were rehydrated for 10 minutes (each) in 95, 75, 50, and 25% methanol in water/DEPC solutions, then 5 minutes in each of three changes of PTW. After incubation in 2.5 µg/ml of Proteinase K in PTW/DEPC for 10 minutes at room temperature, the embryos were washed for 2 minutes with PTW/DEPC. Note that incubation with Proteinase K is optional, and may be omitted if the embryo will be used for immunolabeling. The embryos were then washed for 5 minutes in each of two changes of a fresh TEA solution [0.1M triethanolamine in PTW/DEPC solution: PBS plus 0.1% of Tween 20 and 1% DEPC, pH 7.8]. Without withdrawing the TEA, 2.5 µl of acetic anhydride was added to each ml of TEA. After inverting the tube to mix, then leaving it in gentle agitation at room temperature for 5 minutes, 2.5 µl of acetic anhydride was added to the tube and left to settle for 5 minutes. The embryos were then washed with PTW/DEPC for 5 minutes in each of two changes [this step is omitted if the treatment with proteinase K was not made]. The embryos were fixed with 4% formaldehyde in PTW/DEPC for 20 minutes, then washed with PTW/DEPC, for 5 minutes in each of five changes. The PTW/DEPC was removed, 1 ml of hybridization buffer (HB) was added [5 ml formamide, 2.5 ml SSC solution (175.3 g ClNa, 88.2 g sodium citrate, 0.1% DEPC in 1000 ml twofold-distilled water), 340 µl of 30 mg/ml torula RNA, 20 µl of 50 mg/ml of heparine, 200 µl of Denhardt solution (D) (5 g bovine serum albumin, 5 g polyvinylpyrrolidone-40, and 5 g ficoll 400 in 500 ml of distilled water), 100 µl of 10% Tween 20, 100 µl of 10% CHAPS, 200 µl of 0.5M EDTA pH 8, 1500 µl distilled water], without salmon sperm DNA, diluted ¼ with PTW/DEPC, and shaken for 5 minutes. The fluid was replaced with 500 µl of HB without salmon sperm DNA, incubating at 62°C for 10 minutes. Ten mg/ml of salmon sperm DNA was denatured for 10 minutes at 100°C. The fluid was replaced with 500 µl of HB plus 50 µl of salmon sperm DNA, pre-hybridized for 3 hours (minimum) at 62°C. The fluid was replaced with 250 µl of HB plus 25 µl of salmon sperm DNA, 1 µg/ml of labeled riboprobe was added, and hybridized for 16–18 hours at 60 to 65°C according to probe size.

Second day (anti-DIG antibody incubation)

Solutions of HB and SSC 2X were heated at 62°C. The solution with the probe was removed. Note that used solution may be stored at −20°C for re-use. Each embryo was washed with 2 ml of HB for 10 minutes at 62°C, then washed for 20 minutes in each of three changes of SSC 2X/DEPC at 62°C. From this step onward, it is possible to work in 'not RNase-free' conditions. The embryos were incubated for 30 minutes in SSC 2X with 20 µg/ml of RNase A and 10 U/ml of RNase T1 at 37°C, then washed for 10 minutes with 2 ml of SSC 2X at room temperature, and afterwards for 30 minutes in each of two changes of fresh SSC 0.2 X plus 0.1% DEPC at 62°C. The embryos were washed for 10 minutes in each of two changes of MAB solution [30 ml of 5M NaCl and 16 g maleic acid in 1000 ml of distilled water, adjusted to pH 7.5] at room temperature. The washing solution of each embryo was replaced with 0.5 ml of 2% blocking reagent solution in MAB, then incubated for 15 minutes at room temperature; subsequently, the blocking solution was replaced and incubated for 1 hour at room temperature. The solution was replaced with 0.5 ml of blocking reagent in MAB solution plus the corresponding antibody. If the probe was labeled with digoxigenin, anti-digoxigenin-AP (Roche, Mannheim, Germany) is used at 1/2000 dilution. The incubation was performed for 3 hours at room temperature (or optionally for 16–18 hours at 4°C). Washings for 5–10 minutes in each of three changes of MAB solution were followed by another wash for 16–18 hours at 4°C.

Third day (probe detection)

After additional washings for 30–60 minutes in each of two changes of MAB solution at room temperature, the embryos were incubated for 5 minutes in each of two changes of developing buffer (DB) [100 mg Tween 20 and 120 mg Levamisol in 100 ml of AP buffer (25 ml of 1M Tris pH 9.5, 5 ml of 2.5M MgCl and 5 ml of 5M NaCl in 250 ml of distilled water)]. Then the embryos were transferred to glass containers with 3 ml of DB containing 14 µl/ml of NBT and BCIP substrates and incubated at 37°C in the dark. When the embryos reached the desired density, the solution was replaced with methanol. If the result of hybridization was good, the embryos were hydrated for 10 minutes in each of 95, 75, 50, and 25% methanol in PBS, then with PBS alone. The embryos may be preserved for a long time in 4% paraformaldehyde in PBS.

Whole immunolabeling of cultured chick embryos

The expression of specific mRNAs in the cell does not necessarily mean that the corresponding protein was synthesized and is functional. Therefore, in the experimental approaches, in addition to the detection of the mRNA transcripts by *in situ* hybridization, it was also desirable to attempt to show the resulting protein expression by immunolabeling, a goal that is not easy to reach due to the very low concentration of the functional chemotactic proteins.[10,34]

Obtaining and processing chick embryos

See above, under '*In situ* hybridization'. The DEPC can be omitted if the embryos will be used only for protein immunolabeling.

Immunocytochemistry protocol

Techniques for visualization with fluorescent markers will be briefly described. In the present work, the primary antibodies used were: (1) goat polyclonal anti-SDF-1 (Santa Cruz Biotechnology Cat. no. SC-6193), (2) rabbit polyclonal anti-NT-3 (US Biological lot L5110356 Cat. no. LN 6000-07C), and (3) mouse monoclonal anti-NC-1 neural crest marker.[35] Secondary antibodies were fluoresceine-labeled rabbit anti-goat, rabbit anti-mouse and goat anti-rabbit IgGs.

Briefly, the embryo and extraembryonic membranes were excised, sectioning with fine scissors 5 mm outside the vascular area. Then the embryonic disk was raised by means of a 13 mm diameter filter membrane, washed for 10 minutes in each of three changes of Ringer's saline at room temperature, fixed for 3 hours with 4% paraformaldehyde in Ringer's and piercing the ectoderm with a microneedle to allow antibody penetration. Cell permeabilization, if indicated, was made with 1% Triton X-100 in PBS for 1–2 hours, at room temperature.

The whole cultured chick embryos were then rinsed with Ringer's solution and incubated in a wet chamber at room temperature with blocking solution (1% bovine serum albumin and 1.5% 0.2M Glycine in PBS) three times, 1 hour each.

The embryos were then incubated with the corresponding primary antibody diluted in blocking solution at 4°C, for 12 hours to 4 days, depending on the embryo age. After washing with blocking solution, three changes × 30 minutes each at room temperature, the embryos were incubated with the secondary antibody conjugate with fluorescein isothiocyanate (FITC) for 8 hours to 3 days at 4°C depending on the embryo age. Then the embryos were washed for 15 minutes in each of three changes of PBS. The embryos were post-fixed with 4% formaldehyde in PBS for 15 minutes, and washed for 10 minutes in each of three changes of PBS.

All the previous technical steps were carried out in Petri dishes series or, alternatively, the embryo-filter assembly was mounted into a stainless steel holder device (Cat. no. 3001200, Swinny filter holder 13 mm SS, Millipore, Billerica, MA, USA), and the top end of the filter unit was connected to a three-way stopcock to allow changes of the environment with different fluids.[29]

After a last PBS washing, preparations were mounted with coverslip using anti-bleaching medium and observed with a fluorescence filter for FITC (excitatory filter=450–480 nm and barrier filter= 515 nm). Images were obtained with Olympus CX41 and BX50 microscopes, using an Olympus C7070 digital camera (Olympus Corp., Shinjuku-ku, Tokyo, Japan), or a Hamamatsu C2400 video-camera (Hamamatsu Photonics K.K., Tokyo, Japan), and submitted to image analyses with the SigmaScanPro (SPSS, Chicago, IL, USA) software, according to previous descriptions.[19,21,22]

Neural crest cell cultures

Primary cultures of NCCs were made from chick embryos (*Gallus gallus*, Cobb line) incubated at 38 ± 1°C in a humidified atmosphere up to stage HH 10 to 11.[4,19,21,22,28] Briefly, after cutting and opening the ectoderm, mesencephalic NCCs were obtained by microdissection from the mass of NCCs bilateral to the neural tube, transferred to coverslips precoated with fibronectin[4] and incubated in Petri dishes (35 mm, Sigma Co., St. Louis, MO, USA) with 2 ml of N2 defined medium (N2 basal medium plus

5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine and 30 nM selenium in 100 ml of medium)[19,21,22] supplemented with 10% fetal calf serum (Sigma Chem. Co., St Louis, MO, USA) during 24 hours at 37 ± 0.2°C in 5% CO_2 in air. Applying the careful microdissection technique described, the degree of purity of NCC cultures was constantly near 100%, without neural tube, ectoderm and/or mesoderm contaminants. If some culture contained tissue contaminants, they were detected by phase contrast microscopy and NC-1 immunolabeling[20,35] and consequently discarded.

Evaluation of chemotaxis

A coverslip carrying cultured NCCs was mounted upside down on a modified chemotaxis chamber[22] and perfused with a concentration gradient of SDF-1, NT-3, or Shh (S8406, N1905, and S0191; Sigma Chem. Co., St Louis, MO, USA) at the concentration indicated in Results (or N2 medium=control). Other experiments were carried out with factor samples pre-incubated with 1–2 μg/ml of the corresponding neutralizing antibody anti-SDF-1 (ab9797, Abcam Inc.), anti-NT-3 (N9773, Sigma Chem. Co.) or anti-Shh (S4944, Sigma Chem. Co.), or with heat-inactivated molecules, or in the presence of antibodies against respective receptors (Abcam Inc., Cambridge, MA, USA).

After mounting, the chemotaxis chamber was placed at 37 ± 0.1°C on a video-microscopy system and real-time recorded for 6 hours. Cell contours of each cell were captured and transferred to a computer using a commercial software (SigmaScanPro, SPSS, California, IL, USA) to analyze morphometric, dynamic and several chemotactic parameters applying algorithms developed in our laboratory.[11,20–22] Besides the proportion of migrating cells toward the putative attractants and the chemotactic index, several chemotactic parameters were tested under strict real-time directional criteria, such as the net distance traveled up to gradient, the turning angle of each cell trajectory and the angular bias of the whole cell trajectories.[11,22] All results were coherent, without significant differences among the chemotactic parameters evaluated; consequently, in this report only the chemotactic index expressing the efficient distance traveled by a cell toward an attractant will be shown. It is calculated as the quotient of the net distances parallel to the gradient (X axis) of all the migrating cells divided by the total distance traveled (curvilinear distance).[22] For cells responding to a chemotactic gradient, the value approaches a straight line (i.e. tends to 1), whereas for cells moving randomly the value tends to 0.

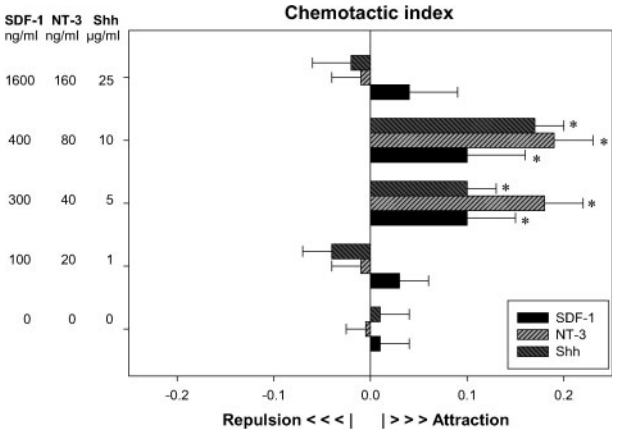

Figure 1 Chemotactic index of NCCs in gradients of stromal cell-derived factor-1 (SDF-1), neurotrophin-3 (NT-3) and sonic hedgehog (Shh) at indicated concentrations (left columns). Chemotaxis is expressed as the quotient between the net distance parallel to the gradient (X axis) and the total distance traveled. Factors pre-incubated with specific antibodies or heat-inactivated factors show values not significantly different to control (see Materials and Methods section). *Significant differences vs control condition.

Statistical treatment

After estimation of the minimal sample size, no fewer than 50 cells and a mean of about 650 cell contours and centroids in each experimental condition repeated in triplicate were studied. Analysis of proportions was performed with a z-test with Yates correction or the corresponding arc sine values, and then a one way ANOVA was conducted, followed by the Scheffé's method for comparing all contrasts, or a Tukey assay, using the SigmaStat (SPSS, California, IL, USA) software. Statistical significance was set at $P \leqslant 0.05$.

Results

Chemotactic response of in vitro NCCs to gradients of diffusible factors

The dynamic behavior of *in vitro* NCCs exposed to concentration gradients of the morphogen Shh, the chemokine SDF-1 and the growth factor NT-3 revealed an active cell response, typical of directional migration (Fig. 1). The chemotactic migration was expressed as the percentage of migrating cells, the net distance traveled up to gradient, the turning angle of the cell trajectory (not shown), and the chemotactic index (Fig. 1). The latter parameter expresses how biased a cell trajectory is, since it is the quotient between the net distance parallel to the gradient and the total distance traveled by the cell. Moreover, chemotactic response was not observed after pre-incubation with specific antibodies against chemotactic factors or their receptors, and these values were not significantly different from those of the heat-inactivated factors or of the control group. Since previous and present *in vitro*

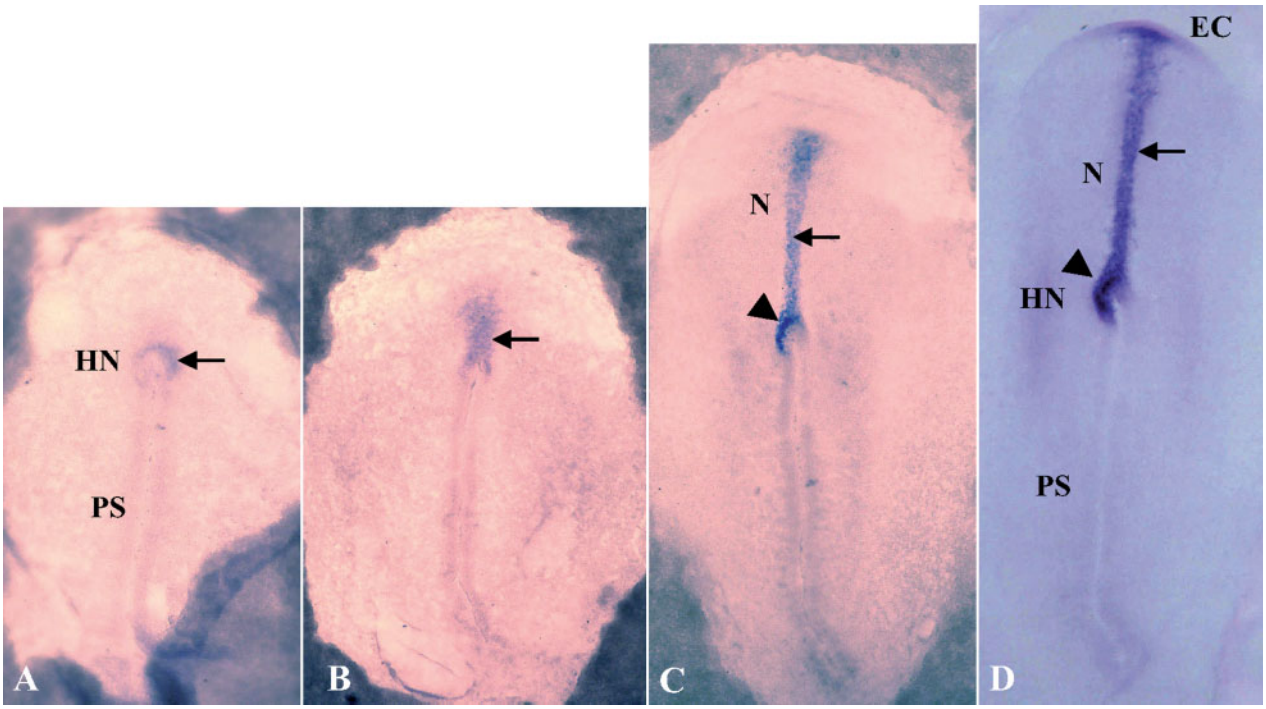

Figure 2 Expression of mRNA of Shh (arrows) in chick embryos. (A) Stage HH 4; (B) Stage HH 5; (C) Stage HH 6; (D) Stage HH 7−. PS, Primitive streak; HN, Hensen's node; N, Notochord; EC, Edge of cephalic ectoderm. Main expression at the left side of Hensen's node (arrowheads).

results support the notion that directional migration of mesencephalic NCCs is modulated by guiding molecules segregated from the optic vesicle region, where the NCCs colonize and differentiate into neurons and glia of the ciliary ganglia, it was necessary to search for the *in situ* location of bioactive molecules.

Expression of sonic hedgehog mRNA

The probe corresponding to Shh allowed us to observe early expression (gastrulation/neurulation) in defined sites of the chick embryo (Figs. 2–4). From stage HH 4 (18–20 hours of development), the expression of Shh was clearly observed in the Hensen's node region (Fig. 2A), without evidence of expression in the primitive streak or in the ectodermic layer. At stage HH 5 (20–22 hours), Shh expression was seen from the cranial domain of Hensen's node toward the notochordal process, which in this stage extends to half the distance between the node and the anterior edge of the embryo disc (Fig. 2B). From stage HH 6 (22–25 hours), the Shh was observed along the notochord, increasing its expression in the left domain of Hensen's node (Fig. 2C). This pattern is maintained in the notochord until the end of the cephalic extension (Fig. 2D). From then, an expansion of the chord expression was observed in the vicinity of the ectoderm covering the cephalic end of the embryo disc, until stage HH 7+ (25–27 hours) (Figs. 2D and 3).

At a more advanced stage (HH 8), Shh expression was intense along the notochord, enlarging progressively from the region at mesencephalic level toward the cephalic end (Fig. 4A), with the largest and strongest expression being at the presumptive prosencephalic region (Fig. 4A, white arrowhead). In embryos at stage HH 12, where the bilateral separation of both optical vesicles occurs, Shh expression is maintained between these two structures, in the middle of prosencephalon (Fig. 4B and C). On the other hand, Shh is persistent along the notochord until its caudal end, and in a more diffuse manner in proportion as they mix with the weak expression of the remaining Hensen's node and the primitive streak (Fig. 4B).

Expression of stromal cell-derived factor-1

The mRNA expression of the chemokine SDF-1 was intense in chick embryos of stage HH 12 (45–48 hours of development), among prosencephalon, mesencephalon and optic vesicle, as well as caudally in the notochord/neural tube floor (Fig. 5A). A detailed study at high magnification showed a dense expression at the basal prosencephalon and neck of the optic vesicle (Fig. 5B). On oblique projection, the heavy expression of SDF-1 embracing the optical stalk was clearly seen (Fig. 5C).

Immunolabeling of NCCs in whole chick embryos at stage HH 12 (Fig. 6) enabled their co-localization with the SDF-1 protein to be seen (Fig. 7). At this stage, mesencephalic NCCs segregate from the neural anlage, as seen in the lower 2/3 of the oval of Fig. 6B,

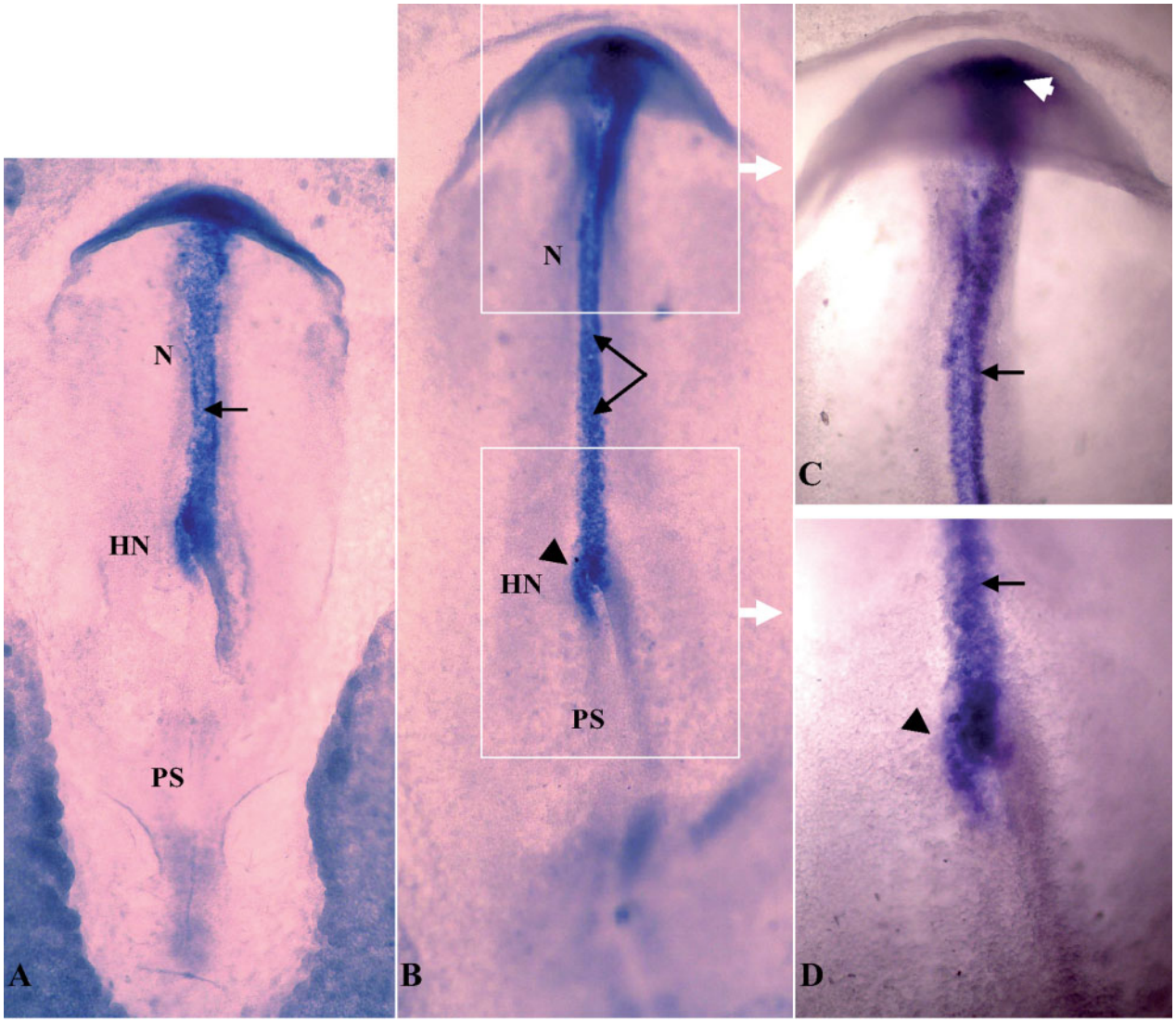

Figure 3 Expression of mRNA of Shh (arrows) on chick embryos. (A) Stage HH 7; (B) Stage HH 7+; (C) and (D) details of B. PS, Primitive streak; HN, Hensen's node; N, Notochord. Main expression at the left side of Hensen's node (black arrowheads) and at the cephalic expansion of the notochord (white arrowhead).

and migrate toward the ventro-medial domain of the optic vesicle (Fig. 6A and B, OV), where they accumulate (upper 1/3 of the oval of Fig. 6B). Immunostaining of the SDF-1 protein was also observed in the domain of the optic stalk (Fig. 7A and B), diffusely extended toward the ventrocaudal region, on both sides of the mesencephalic segment (Fig. 7B). The location of the SDF-1 protein is comparable to the expression of mRNA of SDF-1 (Fig. 5B and C) and the immunolabeling of NCCs (Fig. 6B).

Expression of neurotrophin-3

In chick embryos at stage HH 12, the mRNA of the neurotrophic factor NT-3 expressed on the external surface of the prosencephalon, mesencephalon and rhombencephalon, in the vicinity of the NCC domain (Fig. 8A).

The NT-3 expression showed striking details when observed at high magnification and focusing different dorso-ventral levels from the ectodermic surface toward the ventral zones (Fig. 8C–E). The surface ectoderm showed a very light expression of NT-3, which allowed the dark label to be seen on the subjacent neural tube and optic vesicles (Fig. 8C). The distribution of NT-3 expression was more evident when focusing on the optic vesicle, showing the label from the optic stalk toward the mesencephalic level (Fig. 8D). When focusing on ventral levels, an intense expression label was seen in the region corresponding to the infundibulum and the optic stalk, target fields of the mesencephalic NCCs migrating from the neural tube toward cranial and ventral pathways (Fig. 8E). Immunolabeling of the protein NT-3 was weak but constant, showing a spatiotemporal coincidence with the cephalic fields

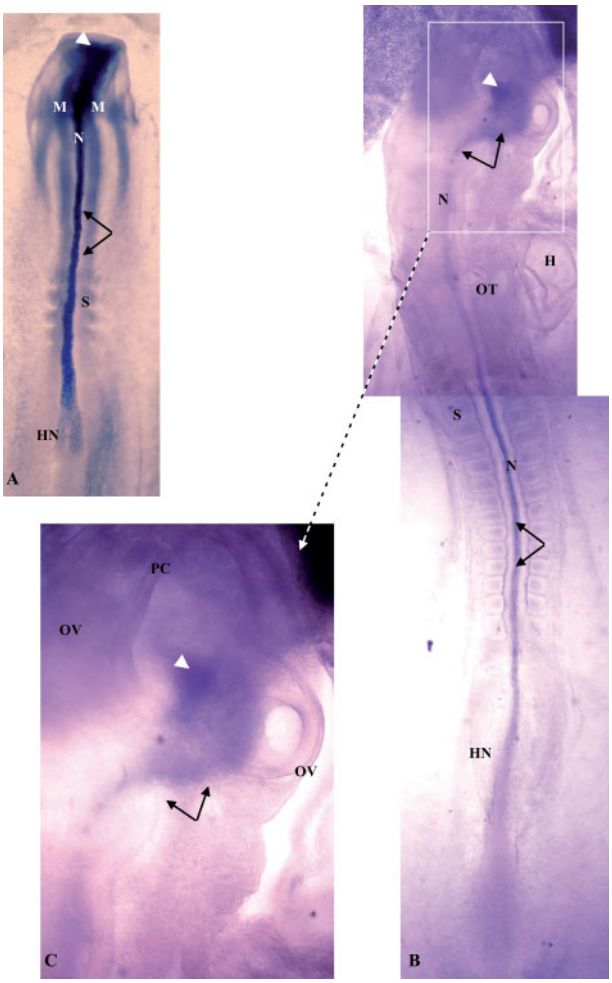

Figure 4 Expression of mRNA of Shh (arrows) on chick embryos. (A) Stage HH 8; (B) Stage HH 12; (C) Details of B. HN, Hensen's node; N, Notochord; S, Somite; M, Mesencephalic neural folds; H, Heart; PC, Edge of the prosencephalic medial closing; OV, Optic vesicle; OT, Otic vesicle. Expanded expression at the cephalic end of the notochord (white arrowheads).

expressing the corresponding mRNA (data not shown).

Discussion

During recent decades, many reports have made it possible to know *where* and *by what ways* embryonic cells distribute. Research results included in the present work address the question of *how* and *why* the embryonic cells orient toward their 'final destiny', moving with high precision among the myriad micro-environmental molecular signals. One way to accomplish this is from the point of view of the chemotactic mechanism, for which it is necessary: (1) to choose a cell population, such as the NCCs, which directionally distributes toward defined target fields throughout the embryo; (2) to have direct experimental evidence about cell directionality toward concentration gradients of known molecules, such as Shh,

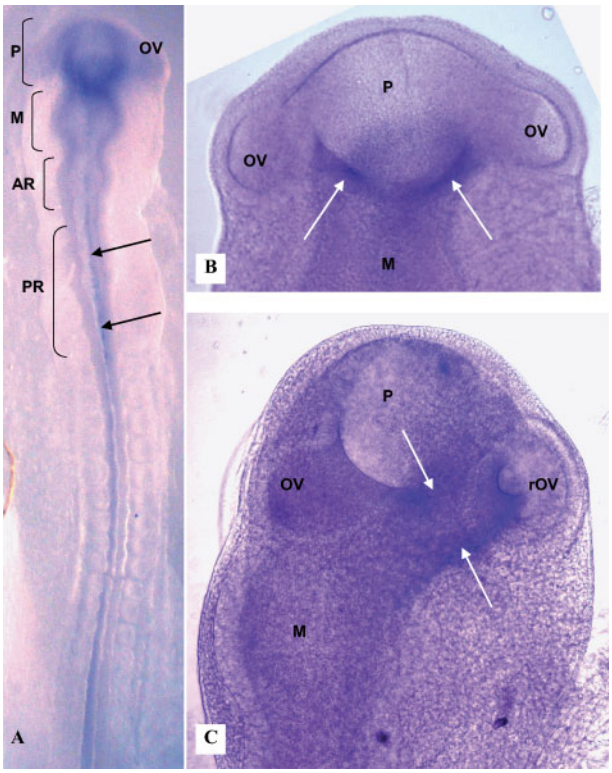

Figure 5 Expression of mRNA of SDF-1 (arrows) on chick embryo. (A) and (B) Stage HH 12, dorsal view. (C) Same embryo, oblique view. P, Prosencephalon; M, Mesencephalon; AR, Anterior rhombencephalon; PR, Posterior rhombencephalon; OV, Optic vesicle; ROV, Right optic vesicle.

SDF-1, and NT-3; (3) to show that these molecules are expressed at the domain colonized by NCCs; and (4) to attempt the *in vitro*/*in vivo* functional blocking of molecules involved in the chemotactic behavior of NCCs. The first two stages were recently reported as results from our laboratory,[19,22,24,25] and the present work deals with the abovementioned 3rd stage, which is essential when experiments on the whole embryo are intended to confirm the *in vivo* chemotactic mechanism of NCCs. One of the methods of choice for localizing bioactive molecules in whole organisms is *in situ* hybridization, which is very useful to detect specific sequences of nucleic acids. When applied on whole mount cultured embryos, it enables the highly sensitive topological location of differential gene expression and detection of mRNA transcripts, with good spatiotemporal resolution.[26,27]

Cephalic NCCs are guided by a chemotactic mechanism

After the chemotaxis analysis under strictly directionality-based criteria,[22] the oriented cell behavior of a subpopulation of NCCs toward Shh, SDF-1, and NT-3 was clearly shown. All chemotaxis parameters involving different expressions of oriented cell migration, such as the proportion of migrating cells, the distance

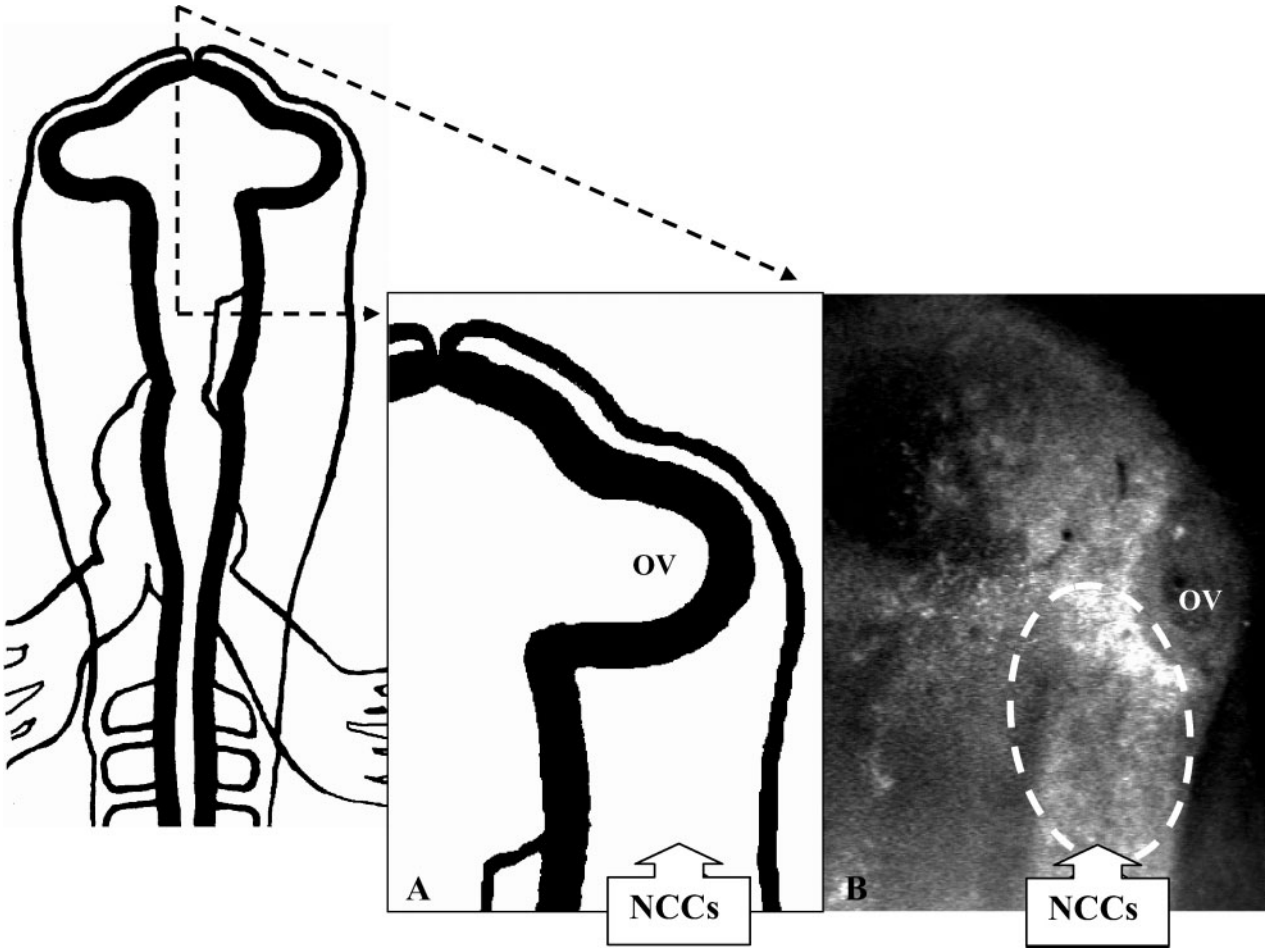

Figure 6 (A) Scheme (and detail) of the cephalic right end of chick embryo at stage HH 12. (B) Immunolabeling of NCCs during migration from the mesencephalic level (lower 2/3 of the oval figure) up to colonization of ocular region where they accumulate (upper 1/3 of the oval figure). OV, Optic vesicle.

traveled toward the attractants, the angular bias of the cell trajectories, and the chemotaxis index, delineated responses with a bell-shaped curve, typical of a concentration-dependent chemotactic behavior.[22,36] Moreover, the oriented cell migration was blocked after pre-treatment of chemotactic factors with specific antibodies against Shh, SDF-1 or NT-3. To our knowledge, the present report provides the first direct evidence that these molecules modify the directionality of a subpopulation of NCCs, inducing dynamic changes typical of oriented migratory cells. This increased persistence of NCCs could participate in the mechanism of optic vesicle colonization, by guiding the cells through their surrounding milieu to invade the target field.

Several molecules and mechanisms were proposed as working for the efficient locomotion of NCCs, from extracellular matrix molecules,[4,37] to cell-to-cell contacts,[5] 'planar cell polarity',[3] and the mechanism of contact inhibition of locomotion.[38] These and other studies, using recent technologies on *in vivo* embryos, provided valuable data for understanding the migration of NCCs.[39] However, although they are important constituents of migratory behavior, they may be not exclusively responsible but rather cooperative factor(s) for oriented cell motility. Our *in vitro* results, obtained with a system that enables the study of NCC subpopulations confronted with concentration gradients of attractants, and excluding other migratory factor(s), clearly showed that chemotaxis, studied under strict real-time directional criteria, may be an essential element of spatiotemporal orientation of NCCs toward specific fields of the embryo body.[22] In addition, in this sensitive process of NCC guidance, it is also reasonable to consider the confluent (also redundant) mechanisms governed by other growth factors and chemokines,[22–25] providing for any failures in the directional behavior of embryonic cells.

Thus, our *in vitro* approach seems to support the idea that diffusible factors emerging from the optic vesicle region play a crucial role in the precise colonization of NCCs. Following this idea, it was

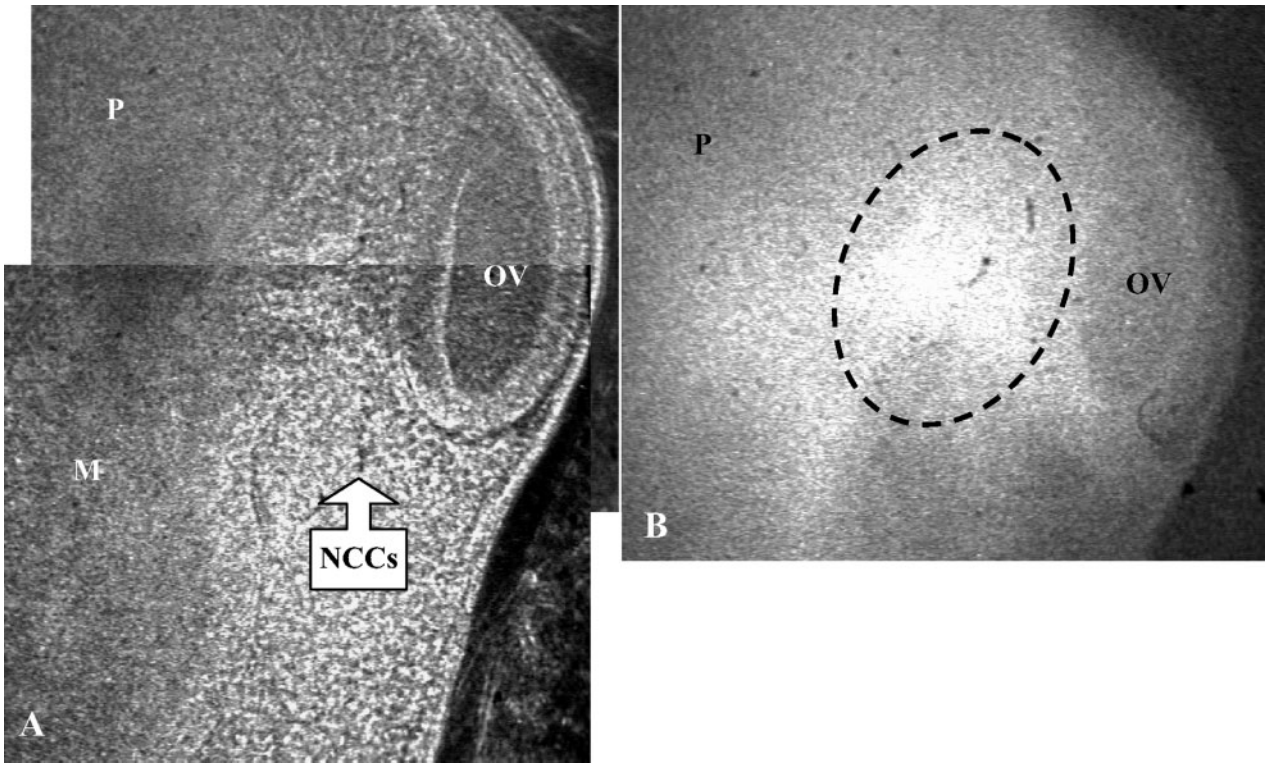

Figure 7 Ocular region of chick embryo at stage HH 12. (A) Phase microscopy; (B) Immunolabeling of SDF-1 (oval) of the same embryo during the neural crest cells (NCCs) colonization of the ocular region. Compare with the expression of mRNA of SDF-1 (Fig. 5B and C) and location of immunolabeled NCCs (Fig. 6B). OV, Optic vesicle; P, Prosencephalon; M, Mesencephalon.

essential to obtain evidence on the real *in situ* expression of the putative chemotactic molecules.

Sonic hedgehog

The Shh protein integrates paracrine factors involved in the complex morphogen family that induce differentiation of cell types and other important functions during early embryo development.[40,41] It is known that Shh expresses in the notochord, participating in ventral neural tube motoneurons and dorsal sensory neuron differentiation,[30,42] as well as in the early establishment of the right/left bilateral axis of the embryo.[43] In the present work, whole embryo *in situ* hybridization also allowed us to confirm Shh expression in a cell population of the left side of Hensen's node as well as along the notochord, from the early stages (HH 6–7+) of development.

It is known that Shh expression is important for optic system development, since the splitting of two bilateral regions of optic vesicles depends on Shh inhibition of Pax6 transcription factor expression in the center of the cephalic neuroectoderm.[40,41] In the present work, from stage HH 6+ to 7−, a heavy Shh expression was observed at the cephalic end of the notochord, with a transverse expansion under the cephalic ectoderm; at stages HH 7 and 8, Shh assumed a funnel shape, widening from the prospective mesencephalic level toward the cranial end of the closing neural tube. The wider zone of Shh expression corresponded to the mean level of the prosencephalon, which had not yet developed the optic vesicles. At the more advanced stage HH 12, persistent Shh expression was observed between both optic vesicles, and it is known that the failure of Shh expression in this domain results in a deficient migration of cells at the middle of the ocular field, resulting in a single cyclopic eye in the center of the face.[44]

It was also shown that Shh signals induce chemotactic behavior of the axonal growth cone[45] and optic nerve oligodendrocyte precursors.[46] Moreover, there is evidence that prenatal ethanol exposure induces craniofacial anomalies associated with bad distribution of NCCs, caused by a loss of Shh expression.[47] Recent results from our laboratory strongly support a close association between the cell distribution of migrating mesencephalic NCCs and the chemotactic gradient of exogenous Shh released by an optic vesicle explant co-culture.[25]

Stromal cell-derived factor-1

The SDF-1 is part of the superfamily of chemokines, a well-known group of chemotactic molecules for hematopoietic cells,[48] also involved in directional migration of primordial germ cells.[49] SDF-1 was also

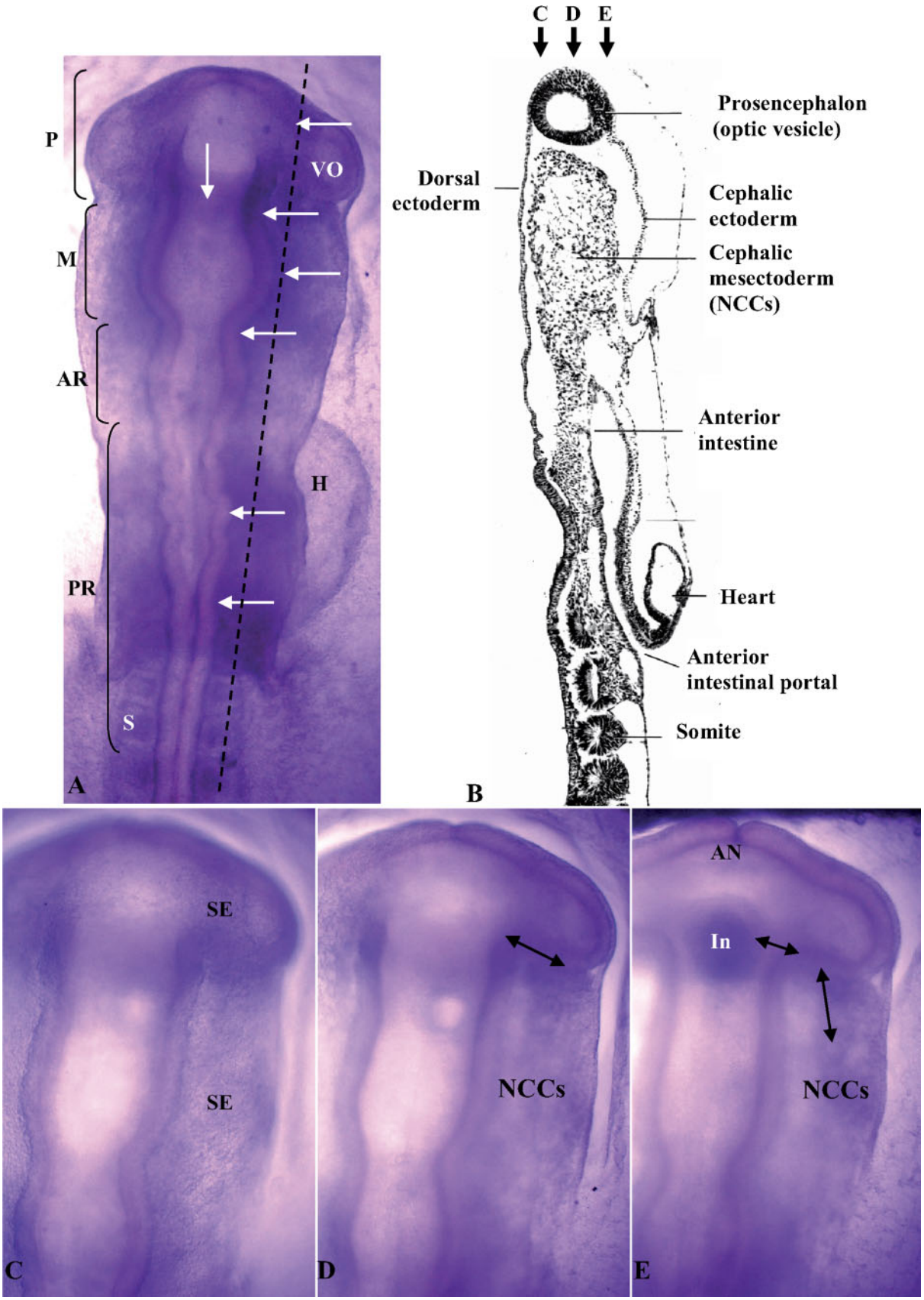

Figure 8 (A) (details on (C), (D), (E)) Expression of mRNA of NT-3 (arrows) of chick embryo at stage HH 12. (B) Longitudinal parasagittal section (indicated on (A) as a broken line), adapted from Bellairs and Osmond (The Atlas of Chick Development, New York: Academic Press; 1998. p. 90). Small arrows at the top localize the optical sections of (C) (ectoderm level), (D) (NCCs middle level) and (E) (NCCs ventral level). P, Prosencephalon; M, Mesencephalon; AR, Anterior rhombencephalon; PR, posterior rhombencephalon; OV, Optic vesicle; H, Heart; S, Somite; SE, Surface ectoderm; NCCs, Neural crest cells; AN, Anterior neuropore; In, Infundibulum.

suggested as a guidance molecule for several embryogenic events,[50] interacting with Shh factor in relation to the proliferation and migration of cerebellar granule cells.[51] In neuronal precursors of the sensorial lateral organ of zebrafish, there is evidence of an SDF-1 chemotactic effect,[52] as well as in progenitors of mice sensory neurons,[14] and sympathetic (NCCs-derived) precursor cells.[15]

Recent evidence from our laboratory [23] and the present results indicated a chemotactic response of mesencephalic NCCs colonizing the optic region after exposure to *in vitro* gradients of SDF-1. Here, to localize the source of this molecule, SDF-1 expression with a spatial distribution coherent with the proposed function was shown. A strong label for SDF-1 mRNA was observed at the base and stalk of the optic vesicle (at the stage when this region is occupied by NCCs). The immunolabeling of SDF-1 protein was also coherent with the hypothesis of NCC chemotaxis, given the observed spatiotemporal coincidence with the NCCs occupying the ocular field and the mRNA expression of SDF-1 in chick embryos of the same developmental stages.

The present results do not indicate the cell population responsible for SDF-1 expression, nor the mechanism of forming and temporally maintaining an extracellular concentration gradient in the optic vesicle neighborhood. Nevertheless, taken as a whole, the data support the hypothesis of chemotactically guided NCCs colonizing discrete sites, and contribute to planning further experiments pointing to the functional blocking of the molecule.

Neurotrophin-3

NT-3 belongs to the family of neural trophic factors, involved in various functional activities at early stages of embryo development as well as in adult organisms. NT-3, like other members of the group, plays a role as a chemotactic factor, stimulating the global motility of neuroblasts and the orientation of the axonal growth cone toward defined target fields in the NCC-derived sensory neurons of the dorsal root ganglia.[53] In chick embryos, the mRNA expression of NT-3 was described from the 4th day of development in the Rathke pouch and infundibulum, while in later stages it is expressed in the brain, mainly in the hippocampus, and in various peripheral tissues.[31]

Recently, we have obtained *in vitro* experimental evidence implicating NT-3 in the directional guidance of mesencephalic NCCs through activation of TrkC and p75 receptors.[24] In the present work, NT-3 mRNA expression and immunolabeling of the protein in earlier chick embryo stages was shown for the first time, in a localized expression in the optic stalk, extended toward the regions lateral to mesencephalic levels, the pathway of NCCs, with a strong medial increase in the infundibulum region.

Although the spatiotemporal distribution of NT-3 mRNA expression is coherent with its proposed chemotactic guidance for NCCs, these results do not permit deductions about the cell population responsible for its expression and secretion. As expected, the immunocytochemistry of NT-3 protein yields a constant but mild label, probably because of the extremely weak concentration of chemotactic proteins in the extracellular micro-environment. It is well known that many chemotactically active molecules exert their directional effect at a very low concentration of $<10^{-9}$M.[10,34] Nevertheless, the present data on localized expression of NT-3 are important for the design of whole embryo experiments applying functional blocking approaches. Moreover, since it was shown that the *in vitro* and *in vivo* ethanol-exposure of NCCs induces selective damage to migratory behavior[20,21] and chemotactic orientation,[23] and that the simultaneous NT-3 treatment repairs ethanol-dependent trophic-survival damage,[19] the present data also open stimulating perspectives toward the search for preventive and/or therapeutic tools in order to protect the abnormal cell distribution induced by prenatal ethanol exposure.

Conclusions

In the present work, the use of suitable probes allowed support and amplification of data on Shh expression reported previously by others. Moreover, the results of expression probes to SDF-1 and NT-3, as well as the corresponding protein immunolabeling, bring objective support to factors probably involved in early chemotactic directional migration of NCCs at the cephalic end of the embryo. This report, showing the *in situ* expression of chemotactic molecules in the whole embryo, significantly contributes to a better comprehension of the mechanism for oriented migration of embryonic cells. These findings are also in line with additional cell-guiding activities for chemokines, trophic factors and morphogens, besides their well-known canonical functions.

Acknowledgements

We thank Mr Joss Heywood for critical reading of the manuscript. We are grateful to Dr A. Carrasco from the Instituto de Biología Celular y Neurociencias (Facultad de Medicina, Universidad de Buenos Aires, Argentina), who also kindly donated the cDNA fragments to produce the Shh probes used in this work. Plasmids with gene sequences to produce chNT-three

probes were kindly donated by Dr F. Hallböök from the Karolinska Institute (Stockholm, Sweden). The cDNA of chDF-1 [GenBank AY451855] was kindly donated by Dr L. R. Read from the University of Guelph (Ontario, Canada). Culture of *E. coli* (DH5alpha lineage) was a gift of Dr R. C. Gehrauh from the CIBICI (Facultad de Ciencias Química. Universidad Nacional de Córdoba, Argentina).

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (FONCYT), the Ministerio de Ciencia y Tecnología de la Provincia de Córdoba and the Secretaria de Ciencia y Tecnología de la Universidad Nacional de Córdoba (Argentina).

References

1 Le Douarin NM, Kalcheim C. The neural crest. 2nd ed. Cambridge: Cambridge University Press; 1999.
2 Kee Y, Hwang BJ, Sternberg PW, Bronner-Fraser M. Evolutionary conservation of cell migration genes: from nematode neurons to vertebrate neural crest. Genes Develop. 2007;21:391–6.
3 Matthews HK, Marchant L, Carmona-Fontaine C, Kuriyama S, Larraín J, Holt MR, *et al.* Directional migration of neural crest cells *in vivo* is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/RhoA. Develop. 2008;135:1771–80.
4 Rovasio RA, Delouvee A, Yamada KM, Timpl JP, Thiery JP. Neural crest cell migration: requirements for exogenous fibronectin and high cell density. J Cell Biol. 1983;96:462–73.
5 Teddy JM, Kulesa PM. *In vivo* evidence for short- and long-range cell communication in cranial neural crest cells. Develop. 2004;131:6141–51.
6 Chen KC, Ford RM, Cummings PT. Cell balance equation for chemotactic bacteria with a biphasic tumbling frequency. J Math Biol. 2003;47:518–46.
7 van Haastert PJ, Keizer-Gunnink I, Kortholt A. Essential role of PI3-kinase and phospholipase A2 in Dictyostelium discoideum chemotaxis. J Cell Biol. 2007;177:809–16.
8 Gómez-Moutón C, Lacalle RA, Mira E, Jiménez-Baranda S, Barber DF, Carrera AC, *et al.* Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. J Cell Biol. 2004;164:759–68.
9 Paratcha G, Ibañez CF, Ledda F. GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream. Mol Cell Neurosci. 2006;31:505–14.
10 Mortimer D, Fothergill T, Pujic Z, Richards LJ, Goodhill GJ. Growth cone chemotaxis. Trends Neurosci. 2008;31:90–8.
11 Fabro G, Rovasio RA, Civalero S, Frenkel A, Caplan R, Eisenbach M, *et al.* Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. Biol Reprod. 2002;67:1565–71.
12 Sun F, Giojalas LC, Rovasio RA, Tur-Kaspa I, Sanchez R, Eisenbach M. Lack of species-specificity in mammalian sperm chemotaxis. Dev Biol. 2003;255:423–7.
13 Guidobaldi HA, Teves ME, Unates DR, Anastasıa A, Giojalas LC. Progesterone from the cumulus cells is the sperm chemoattractant secreted by the rabbit oocyte cumulus complex. PLoS ONE 2008;3:e3040.
14 Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci. 2005;25:3995–4003.
15 Kasemeier-Kulesa JC, McLennan R, Romine MH, Kulesa PM, Lefcort F. CXCR4 controls ventral migration of sympathetic precursor cells. J Neurosci. 2010;30:13078–88.
16 Kubota Y, Ito K. Chemotactic migration of mesencephalic neural crest cells in the mouse. Dev Dyn. 2000;217:170–9.
17 Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Develop. 2002;129:5151–60.
18 Jones MC. The neurocristopathies: reinterpretation based upon the mechanism of abnormal morphogenesis. Cleft Palate J. 1990;27:136–40.
19 Jaurena MB, Carri NG, Battiato NL, Rovasio RA. Trophic and proliferative perturbations of *in vivo*/*in vitro* cephalic neural crest cells after ethanol exposure are prevented by Neurotrophin-3. Neurotoxicol Teratol. 2011;33:422–30.
20 Rovasio RA, Battiato NL. Role of early migratory neural crest cells in developmental anomalies induced by ethanol. Int J Dev Biol. 1995;39:421–2.
21 Rovasio RA, Battiato NL. Ethanol induces morphological and dynamic changes on *in vivo* and *in vitro* neural crest cells. Alcohol Clin Exp Res. 2002;26:1286–98.
22 Rovasio RA, Faas L, Battiato NL. Insights into Stem Cell Factor chemotactic guidance of neural crest cells revealed by a real-time directionality-based assay. Eur J Cell Biol. 2012;91:375–90.
23 Jaurena MB. The chemokine Stromal cell-Derived Factor-1 participate in the oriented migration of neural crest cells in normal ontogenesis and exposed to ethanol. PhD thesis, Biology Sciences School, National University of Cordoba, Argentina, 2011.
24 Zanin JP. Neurotrophin-3 involvement in the modulation mechanism of directional migration of neural crest cells. PhD thesis, Biology Sciences School, National University of Cordoba, Argentina, 2011.
25 Tolosa EJ. Embryonic cell distribution: Involvement of *Sonic Hedgehog-Gli* signals in the oriented motility of neural crest cells. PhD thesis, Biology Sciences School, National University of Cordoba, Argentina, 2012.
26 Sawitzke AL, Chapman SC, Bleyl SB, Schoenwolf GC. Improvements in histological quality and signal retention following *in situ* hybridization in early chick embryos using plastic resin and recolorization. Biotech Histochem. 2005;80:35–41.
27 Streit A, Stern CD. Combined whole-mount *in situ* hybridization and immuno-histochemistry in avian embryos. Methods. 2001;23:339–44.
28 Hamburger V, Hamilton H. A series of normal stages in the development of the chick embryo. J Morphol. 1951;88:49–82.
29 Battiato NL, Paglini MG, Salvarezza SB, Rovasio RA. Method for treatment and processing of whole chick embryos for autoradiography, immuno-cytochemistry and other techniques. Biotech Histochem. 1996;71:286–8.
30 Riddle RD, Johnson RL, Laufer E, Tabin C. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell. 1993;75:1401–16.
31 Hallbook F, Ibañez CF, Ebendal T, Persson H. Cellular localization of Brain-derived Neurotrophic Factor and Neurotrophin-3 mRNA expression in the early chicken embryo. Eur J Neurosci. 1993;5:1–14.
32 Read LR, Cumberbatch JA, Buhr MM, Bendall AJ, Sharif S. Cloning and characterization of chicken stromal cell derived factor-1. Dev Comp Immunol. 2005;29:143–52.
33 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning – A laboratory manual. 2nd ed. New York: Cold Spring Harbor Lab Press; 1989.
34 Yu SR, Burkhardt M, Nowak M, Ries J, Petrasek Z, Scholpp S, *et al.* Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules. Nature. 2009;461:533–7.
35 Vincent M, Duband JL, Thiery JP. A cell surface determinant expressed early on migrating avian neural crest cells. Brain Res. 1983;285:235–8.
36 Gore BB, Wong KG, Tessier-Lavigne M. Stem cell factor functions as an outgrowth-promoting factor to enable axon exit from the midline intermediate target. Neuron. 2008;57:501–10.
37 Lock JG, Wehrle-Haller B, Stromblad S. Cell–matrix adhesion complexes: master control machinery of cell migration. Sem Cancer Biol. 2008;18:65–76.
38 Carmona-Fontaine C, Matthews HK, Kuriyama S, Moreno M, Dunn GA, Parsons M, *et al.* Contact inhibition of locomotion *in vivo* controls neural crest directional migration. Nature. 2008;456:957–61.
39 Kulesa PM, Bailey CM, Kasemeier-Kulesa JC, McLennan R. Cranial neural crest migration: new rules for an old road. Dev Biol. 2010;344:543–54.

40 Gilbert SF. Developmental Biology. 7th ed. Sunderland, MA: Sinauer Ass. Inc.; 2005.
41 Wolpert L, Jessell T, Lawrence P, Meyerowitz E, Robertson E, Smith J. Principles of Development. 3rd ed. New York: Oxford University Press Inc.; 2007.
42 Yamada T, Pfaff SL, Edlund T, Jessell TM. Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. Cell. 1993;73:673–86.
43 Rodríguez-Esteban C, Capdevila J, Economides AN, Pascual J, Ortiz A, Izpisua-Belmonte JC. The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. Nature. 1999;401:243–51.
44 Chiang C, Litingtung Y, Lee E, Young KE, Corden JE, Westphal H, *et al.* Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996;383:407–13.
45 Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M. The morphogen Sonic Hedgehog is an axonal chemoattractant that collaborates with Netrin-1 in midline axon guidance. Cell. 2003;113:11–23.
46 Merchán P, Bribian A, Sánchez-Camacho C, Lezameta M, Bovoleta P, de Castro F. Sonic hedgehog promotes the migration and proliferation of optic nerve oligodendrocyte precursors. Mol Cell Neurosci. 2007;36:355–68.
47 Ahlgren SC, Thakur V, Bronner-Fraser M. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. Proc Natl Acad Sci USA. 2002;99:10476–81.
48 Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos J. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med. 1997;185:111–20.
49 Stebler J, Spieler D, Slanchev K, Molyneaux KA, Richter U, Cojocaru V, *et al.* Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12. Dev Biol. 2004;272:351–61.
50 McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol. 1999;213:442–56.
51 Klein RS, Rubin JB, Gibson HD, DeHaan EN, Alvarez-Hernandez X, Segal RA, *et al.* SDF-1α induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. Development. 2001;128:1971–81.
52 Ghysen A, Dambly-Chaudiere C. Development of the zebrafish lateral line. Curr Opin Neurobiol. 2004;14:67–73.
53 Paves H, Saarma M. Neurotrophins as *in vitro* growth cone guidance molecules for embryonic sensory neurons. Cell Tissue Res. 1997;290:285–97.