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Xenopus cadherin-11 restrains cranial neural crest migration and influences neural crest specification

Annette Borchers[1], Robert David[2] and Doris Wedlich[2,*]

[1]Department of Genetics, Stanford University, Stanford, CA 94305-5120, USA
[2]Department of Biochemistry, University of Ulm, Germany
*Author for correspondence at present address: Institute of Zoology II, University Karlsruhe (e-mail: doris.wedlich@zi2.uni-karlsruhe.de)

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SUMMARY

Cranial neural crest (CNC) cells migrate extensively, typically in a pattern of cell streams. In *Xenopus*, these cells express the adhesion molecule Xcadherin-11 (Xcad-11) as they begin to emigrate from the neural fold. In order to study the function of this molecule, we have overexpressed wild-type Xcad-11 as well as Xcad-11 mutants with cytoplasmic (ΔcXcad-11) or extracellular (ΔeXcad-11) deletions. Green fluorescent protein (GFP) was used to mark injected cells. We then transplanted parts of the fluorescent CNC at the premigratory stage into non-injected host embryos. This altered not only migration, but also the expression of neural crest markers.

Migration of transplanted cranial neural crest cells was blocked when full-length Xcad-11 or its mutant lacking the β-catenin-binding site (ΔcXcad-11) was overexpressed. In addition, the expression of neural crest markers (*AP-2*, *Snail* and *twist*) diminished within the first four hours after grafting, and disappeared completely after 18 hours. Instead, these grafts expressed neural markers (2G9, *nrp-1* and *N-Tubulin*). β-catenin co-expression, heterotopic transplantation of CNC cells into the pharyngeal pouch area or both in combination failed to prevent neural differentiation of the grafts.

By contrast, ΔeXcad-11 overexpression resulted in premature emigration of cells from the transplants. The *AP-2* and *Snail* patterns remained unaffected in these migrating grafts, while *twist* expression was strongly reduced. Co-expression of ΔeXcad-11 and β-catenin was able to rescue the loss of *twist* expression, indicating that Wnt/β-catenin signalling is required to maintain *twist* expression during migration.

These results show that migration is a prerequisite for neural crest differentiation. Endogenous Xcad-11 delays CNC migration. Xcad-11 expression must, however, be balanced, as overexpression prevents migration and leads to neural marker expression. Although Wnt/β-catenin signalling is required to sustain *twist* expression during migration, it is not sufficient to block neural differentiation in non-migrating grafts.

Key words: Cadherin, Neural crest, Migration, *Xenopus*

INTRODUCTION

Cadherins are a multigene family of glycoproteins that mediate Ca^{2+}-dependent cell-cell adhesion. They form adhesion complexes with catenins through their cytoplasmic domains. The adhesive strength of these complexes depends upon homophilic binding of the extracellular cadherin domain, and their anchorage to the cytoskeleton, which is mediated by β/α-catenin or plakoglobin/α-catenin bridges (Kemler, 1993; Takeichi, 1995). There is also increasing evidence that lateral clustering of cadherins occurs. In those cases, binding of $p120^{ctn}$ to the intracellular juxtamembrane domain (Navarro et al., 1995; Yap et al., 1998; Ozawa and Kemler, 1998; Ohkubo and Ozawa, 1999; Thoreson et al., 2000) or chemical properties of the transmembrane domain itself (Huber et al., 1999) modulate the adhesive strength.

It was previously assumed that cadherin function is restricted to non-migrating, predominantly polarised epithelial tissues where cadherins are found in adherens junctions (Kemler, 1992). The most thoroughly studied member of the cadherin gene superfamily, *E-cadherin*, was characterised as a tumour suppressor gene because loss of its expression correlated with increased invasiveness of tumours (Birchmeier and Behrens, 1994). Interestingly, downregulation of cadherin expression in early development correlates with the start of migration. For example, when chicken neural crest cells delaminate from the neural folds, Ca^{2+}-dependent adhesion decreases, and N-cadherin and c-cad6B are downregulated (Newgreen and Gooday, 1985; Akitaya and Bronner-Fraser, 1992; Nakagawa and Takeichi, 1995).

However, the identification of the type II classical cadherins, cadherin-11, cadherin-6 and cadherin-7 (Tanihara et al., 1994; Hoffmann and Balling, 1995; Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998; Inoue et al., 1997; Hadeball et al., 1998; Vallin et al, 1998), and of the protocadherins PAPC and AXPA (Kim et al., 1998) led to an exciting discovery: these cadherins are upregulated in migrating cells, such as neural crest cells, as well as in invasive tumour cells and mesodermal

cells undergoing convergent extension movements. Still, despite the correlation of their expression profiles with cell movement, little is known about their function in cell migration or cell differentiation.

The cranial neural crest (CNC) is ideal to study these questions because it exhibits highly migratory cell behaviour while simultaneously undergoing cell specification. At the beginning of the 20th century, classical grafting and ablation experiments in amphibians had already revealed that these cells gave rise to craniofacial cartilage, the peripheral nervous system (PNS) and pigment cells (Landacre, 1921; Stone, 1921; Raven, 1933). The identification of neural crest marker genes (Hopwood et al., 1989; Winning et al., 1991; Essex et al., 1993) and novel molecular labelling and microscopic techniques allowed the confirmation of these morphological observations in different organisms (Le Dourain, 1982; Sadaghiani and Thiebaud, 1987; Hall and Hörstadius, 1988).

The presence of some common progenitor cells for the different neural crest derivatives at the migratory stage is still in discussion (Groves and Bronner-Fraser, 1998; Mayor et al., 1998; LaBonne and Bronner-Fraser, 1999). Single cell tracking (Collazo et al., 1993) and neural crest cell culture studies (LeDourain and Smith, 1988; Anderson et al., 1997) support the existence of multipotent neural crest progenitors that become committed to different fates during migration. There is strong evidence that neural crest is induced by inhibition of BMP, followed by activation of canonical Wnt/β-catenin or e/bFGF signalling (Mayor et al., 1995; Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). This leads to activation of marker genes *Slug/Snail*, *twis*t and *AP-2* at the premigratory stage. Recent reports (Carl et al., 1999; LaBonne and Bronner-Fraser, 2000) have revealed a function for *Slug* in CNC migration. However, neither the control of movement at the cellular level nor the process of cell specification during migration is understood.

We have investigated the function of Xcad-11, which is expressed in migrating CNC cells. We found that endogenous Xcad-11 expression restrains cranial neural crest migration. However, preventing the migration of CNC cells results in a change from CNC marker expression (*AP-2*, *Snail* and *twist*) to neural marker expression (2G9, *nrp-1* and *N-Tubulin*). This switch was independent of both Wnt/β-catenin signalling and transplant localisation, suggesting that an increase in cell adhesion promotes neural differentiation. Wnt/β-catenin signalling, however, was found essential for *twist* expression in migrating CNC.

MATERIALS AND METHODS

Plasmid construction and in vitro transcription

Xcad-11 constructs (Hadeball et al., 1998) were Myc-tagged by cloning into pcDNA3.1/Myc-His-A (Invitrogen, Groningen, Netherlands). For Xcad-11 amplification, primers were designed so that the stop codon was replaced by *Xba*I and *Kpn*I restriction sites (Xcad-11 forward, 5′-GAGTCTAGATCGGCACGAGCTGGAGT-3′; Xcad-11 reverse, 5′-GTGGTACCAGAATCATCTTCACAAGTGTC-3′). Amplification and ligation were carried out according to Peqlab manual instructions. To clone the cytoplasmic deletion mutant (ΔcXcad-11) that lacked the C-terminal 426 bp, a Xcad-11 subclone in pCRS (Hadeball et al., 1998) was cut with *Xho*I and *Sma*I, and ligated into pcDNA3.1/Myc-His-A cleaved with *Xho*I and *Eco*RV. The plasmid encoding Myc-tagged GFP (green fluorescent protein) was kindly provided by Dr Thomas Joos. Capped mRNAs were synthesised in vitro from linearised plasmids using SP6 and T7 mMessage mMACHINE kit (Ambion, Austin, TX).

GST-pull-down assay

Xcad-11 and ΔcXcad-11 protein were expressed from circular plasmids using the transcription and translation kit (TNT) from Promega (Mannheim, Germany) according to the manufacturer's instructions. GST-β-catenin harbouring amino acids 1-284 of β-catenin (Bauer et al., 1998) was expressed in *Escherichia coli* XL-1-blue. Protein expression and pull-down assay were performed as stated previously (Giehl et al., 2000). After SDS-PAGE, the precipitated protein was detected by the monoclonal 9E10 Myc antibody (10 hours, 4°C), and peroxidase-coupled goat anti-mouse antiserum (2 hours, room temperature). Immunoreactive proteins were visualised using the ECL™ western blotting detection system (Amersham, Braunschweig, Germany).

Injection of *Xenopus laevis* embryos

In vitro transcribed mRNA of Xcad-11 (250 pg, 0.6 ng, 0.8 ng, 1 ng, 1.6 ng), ΔeXcad-11 (250 pg, 0.8 ng, 1 ng, 2.3 ng) and ΔcXcad-11 (0.8 ng, 1 ng, 2.5 ng) were co-injected with 100 pg GFP-RNA into one blastomere of a two-cell stage embryo. Embryos were obtained by in vitro fertilisation, cultivated and injected as described previously (Geis et al., 1998), and staged according to the normal table of Nieuwkoop and Faber (Nieuwkoop and Faber, 1975). Embryos at stage 14 exhibiting GFP-fluorescence were sorted in terms of left or right side fluorescence using an Olympus epifluorescence microscope. Embryos were used either for transplantation or further cultivated until stage 28 and analysed by whole-mount in situ hybridisation.

Transplantation assay

Transplantation of CNC was performed as previously described (Borchers et al., 2000). To trace the transplanted cells Myc-tagged GFP-RNA was injected into one blastomere of two-cell embryos**.** The epidermis covering the cranial neural crest area was peeled off from the GFP-positive side. Part of the underlying CNC was removed at the premigratory stage and inserted in an uninjected control, the host embryo, which was treated accordingly. Transfer of neuroepithelial cells was avoided, which was controlled by in situ hybridisation.

The migration pattern of transplanted embryos was analysed by GFP fluorescence from stage 14 to 48 using an Axiophot microscope (Zeiss, Jena, Germany), and documented on Kodak Ektachrome 160T film. To compare the velocity of ΔeXcad-11-expressing and GFP-control transplants, 111 transplants were prepared using three egg batches, which were continuously monitored over a timespan of 48 hours. The migration patterns of transplants exhibiting migration 18 hours after grafting (86% of the GFP and 85% of the ΔeXcad-11 transplants) were compared immediately after transplant healing and at later time points. For further analysis, transplanted embryos of different stages were fixed for 2 hours (room temperature) in 3.7% formaldehyde in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$), and used for whole-mount in situ hybridisation or immunohistochemistry. The transplants were identified in transverse sections by detection of the Myc-tagged GFP protein (monoclonal 9E10 Myc antibody).

Whole-mount in situ hybridisation and immunohistochemistry

Standard and double-staining whole-mount in situ hybridisation were performed according to Hollemann et al. (Hollemann et al., 1999). In the case of AP-2/Snail double in situ hybridisation, colour images were taken immediately after Fast Red staining for AP-2. After removing the red signal by washing in 100% ethanol, the embryos were incubated in digoxigenin antibody and stained for Snail with NBT/BCIP. The following plasmids were used to generate antisense

probes: *AP-2* (Winning et al., 1991), *Snail* (Essex et al., 1993), *twist* (Hopwood et al., 1989), NeuroD (Lee et al., 1995), sox2 (Streit et al., 1997), nrp-1 (Knecht et al., 1995), N-Tubulin (Richter et al., 1988), sox3 (Zygar et al., 1998) and Xcadherin-6 (David and Wedlich, 2000). The template for the neural cell adhesion molecule (N-CAM; Kintner and Melton, 1987) antisense probe was generated via our recently described PCR approach (David and Wedlich, 2001) from *Xenopus* stage 30 total cDNA. Primers were: N-CAM up, 5′- GTCAAGTAAGCGGAGAAGCC-3′; T3/N-CAM lo, 5′-AATTAACCCTCACTAAAGGG-TCCATCCTCAATTGGTTCAC-3′. The Xcad-11 whole-mount probe is directed against base pairs 40-1222, and ranges from the untranslated region to the EC3 domain. The plasmid was linearised with *Sac*I and transcribed using T7 polymerase. All antisense probes were generated from linearised plasmids using the SP6 or T7 transcription Kits (Boehringer, Mannheim, Germany) with DIG RNA Labeling Mix (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. The fluorescein-labelled *twist* probe was created using the Fluorescein RNA Labeling Mix (Boehringer, Mannheim, Germany). The embryos were either examined as whole mounts or serially sectioned, and immunohistochemically analysed. Sectioning, immunohistochemistry and confocal analysis were performed as described previously (Borchers et al., 2000).

RNA purification and RT-PCR

Total RNA was isolated from half heads of stage 28 embryos using the Purescript kit from Biozym (Oldendorf, Germany). For reverse transcription, SUPERSSCRIPT^{TM} RNase H^{-} Reverse Transcriptase (GibcoBRL, Karlsruhe, Germany) was used, and PCR was performed as described in the manual. The primers corresponding to *twist* (Hopwood et al., 1989) and *H4* (Gradl et al., 1999a) were used as previously described. Primers for *AP-2* and *Snail* were as follows: *AP-2* forward, 5′-CTCAATCCCA-ACGAGGTGTTC-3′; *AP-2* reverse, 5′-CAGAAT-AGGATTTGGTCTGGAG-3′; *Snail* forward, 5′-GTGTGTATCACTATTGGGTAGG-3′; *Snail* reverse 5′-TGTCTTTGTGATCATCATTGGG-3′.

RESULTS

The function of Xcad-11 in CNC migration was analysed by neural crest transplantation

Previously, we have shown that the mandibular, hyoid and branchial stream of neural crest cells in *Xenopus* express *Xcad-11* after emigration from the neural fold (Hadeball et al., 1998). To study the function of Xcad-11 in neural crest cell migration, two deletion mutants of Xcad-11 were constructed (Fig. 1A). The extracellular deletion (ΔeXcad-11) removes 72 amino acids between the EC1 and EC2 domains. This includes the QAV homophilic binding motif, which is analogous to the HAV motif in classical type I cadherins. The other deletion construct (ΔcXcad-11) has a truncation of 140 amino acids in the cytoplasmic tail that results in the loss of the β-catenin binding site. Loss of this site was confirmed by GST/β-catenin pull-down assays. Thus, while full-length protein was able to

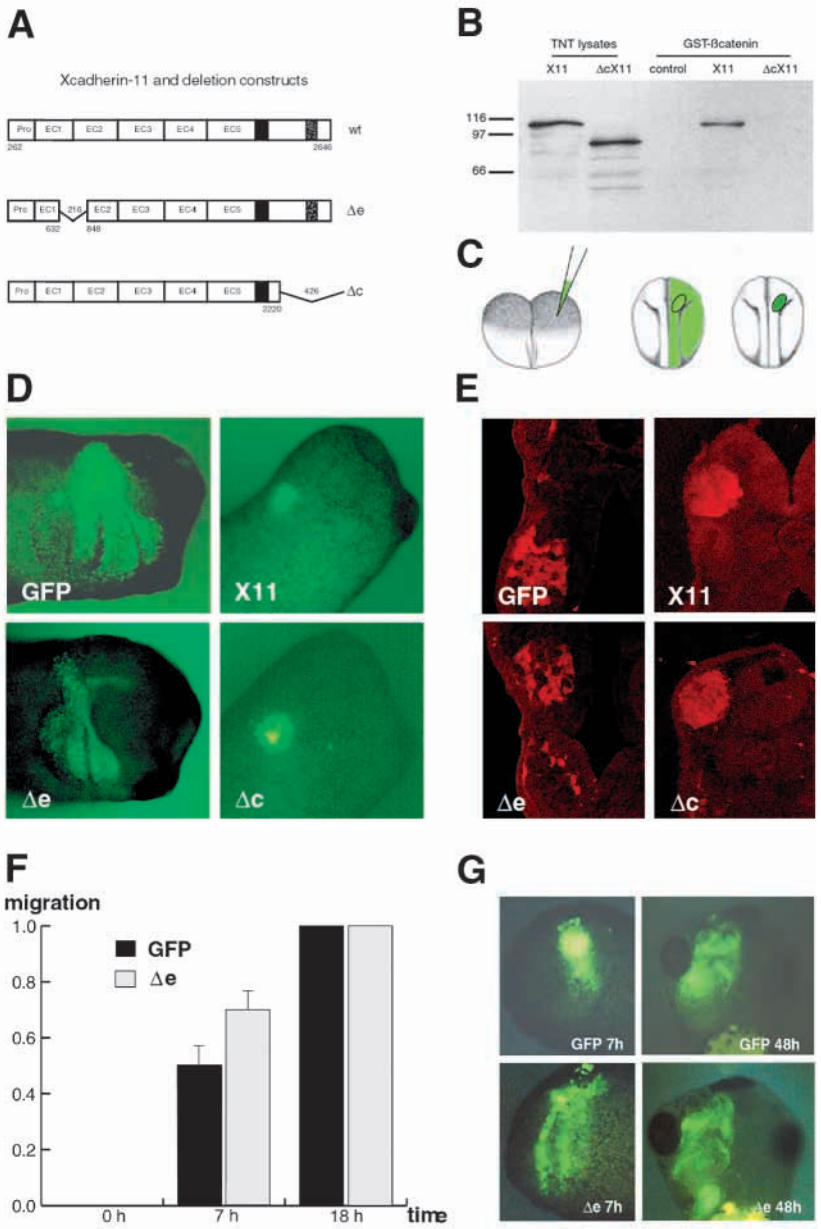

Fig. 1. The extracellular Xcadherin-11 domain regulates adhesion of CNC cells in the transplantation assay, independently of β-catenin-binding. (A) Wild-type and Xcad-11 deletion constructs (black, transmembrane segment; dots, β-catenin-binding site). (B) GST-β-catenin pull-down assay. Western blot showing TNT lysates of full-length (X11pcDNA3.1/Myc-His-A) and cytoplasmic-deleted Xcad-11 (ΔcX11pcDNA3.1/Myc-His-A), all stained with 9E10 Myc antibody (left). Only the full-length Xcad-11 binds the GST-β-catenin fusion protein (right). (C) Transplantation assay. (D) Comparison of cranial neural fold transplants overexpressing different Xcad-11 constructs. Migratory phenotype analysed by GFP fluorescence 18 hours after transplantation. (E) Confocal analysis of transverse transplant sections stained with 9E10 Myc antibody. (F) Transplants expressing the extracellular deletion mutant, ΔeXcad-11 (Δe), started migration earlier than GFP controls. The graph illustrates the comparison of 49 migrating GFP with 46 migrating ΔeXcad-11 (Δe) grafts 0, 7 and 18 hours after transplantation. (G) Lateral views of a transplant expressing ΔeXcad-11 7 hours after transplantation, showing farther migration compared with the GFP control (left). Dorsal views of the same grafts show no differences after 48 hours (right).

bind β-catenin, the cytoplasmic mutant was not able to do so (Fig. 1B).

The different Xcad-11 constructs were tested in a transplantation assay (Borchers et al., 2000). In vitro transcribed wild-type or mutant Xcad-11 RNA was co-injected with Myc-tagged green fluorescence protein (GFP) RNA into one blastomere of two-cell stage *Xenopus laevis* embryos. Before onset of neural crest migration, part of the GFP-positive CNC was transplanted into uninjected GFP-negative host embryos (Fig. 1C). As controls, CNC grafts from embryos injected with only GFP-RNA were used. The migration behaviour of the transplants was evaluated by examining whole mounts for GFP fluorescence 18 hours after grafting.

Overexpression of wild-type or cytoplasmically truncated Xcad-11 (ΔcXcad-11) inhibits migration of CNC cells

Xcad-11 overexpression led to inhibition of CNC cell migration in a dose-dependent manner (Fig. 1D, Table 1). Injection of 1 ng of full-length Xcad-11 RNA resulted in inhibition of migration in 34% of the transplants. Higher doses of injected full-length RNA completely blocked migration but were lethal in the majority of transplanted embryos (e.g. 1.6 ng Xcad-11 RNA, Table 1). Surprisingly, injecting ΔcXcad-11 RNA lacking the β-catenin binding site also blocked migration (Fig. 1D, Table 1). This mutant was less toxic to embryos, allowing the injection of higher RNA doses (2.5 ng). When non-migrating grafts were analysed in transverse sections, the majority of cells were tightly clustered in close proximity to the brain (Fig. 1E). No differences in cell shape or cell behaviour were seen between full-length or ΔcXcad-11 expressing grafts. These results demonstrate that Xcad-11 confers adhesiveness to the injected cells independently of β-catenin.

ΔeXcad-11-expressing transplants start migration earlier than GFP control transplants

As the homophilic binding site is deleted in the extracellular Xcad-11 mutant (ΔeXcad-11, Fig. 1A), we expected a decrease in cell adhesion. Grafts expressing ΔeXcad-11 show a migration pattern similar to that of the GFP control (Fig. 1D). Transverse sections revealed that ΔeXcad-11-expressing cells migrate as a cohort of loosely associated cells indistinguishable from the GFP controls (Fig. 1E). However, when ΔeXcad-11 and GFP control transplants were continuously monitored over the first 18 hours, we observed that cells from ΔeXcad-11-expressing transplants emigrated earlier than those of the GFP controls (Fig. 1F,G). The strongest effect was observed 4 to 7 hours post-grafting, when 70% of the ΔeXcad-11-expressing transplants (n=46) showed cell emigration, while 50% of the controls (n=49) were still as compact as at the beginning of the experiment (Fig. 1F). After 18 hours, however, the GFP controls showed the same 100% migration as the ΔeXcad-11-expressing grafts. The ΔeXcad-11-expressing grafts were indistinguishable from the controls after 48 hours when the cranial crest gave rise to cartilage (Fig. 1F,G).

We also co-injected ΔeXcad-11 with full-length Xcad-11 RNA, and found that inhibition of migration by Xcad-11 was partially restored (Table 1). In contrast, co-expression of ΔcXcad-11 with wild-type RNA led to an increase in the non-migrating phenotype (Table 1). Thus, ΔeXcad-11 acts in a dominant-negative manner in terms of adhesiveness, while ΔcXcad-11 behaves like the wild-type cadherin.

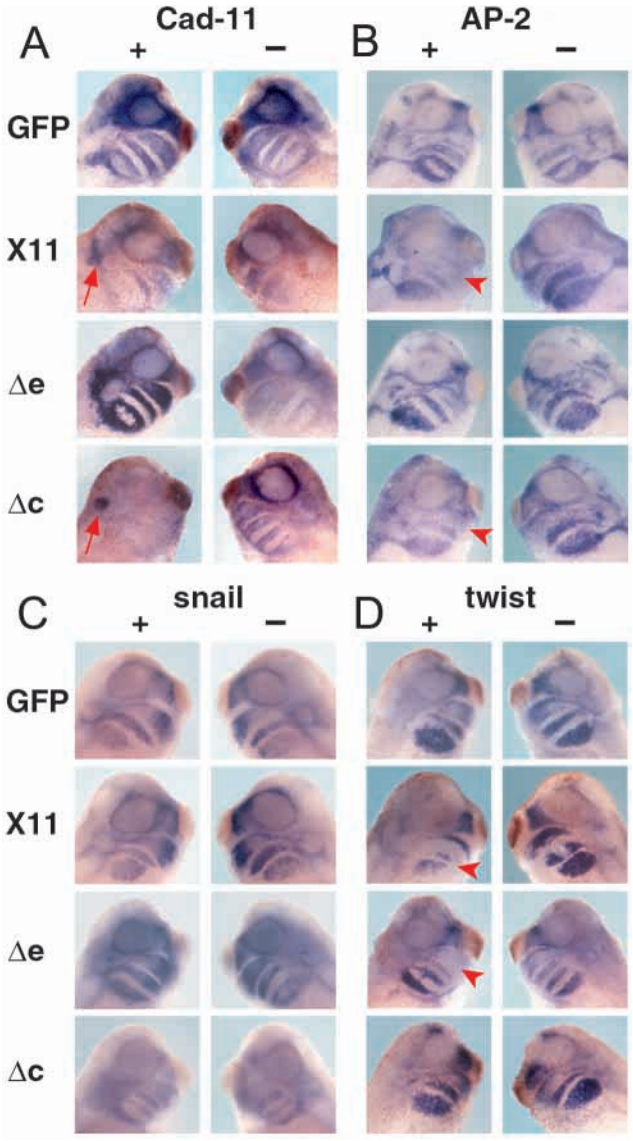

Fig. 2. Migration behaviour controlled by Xcadherin-11 affects specific neural crest markers differently. (A) Xcad-11, (B) AP-2, (C) Snail, and (D) twist whole-mount in situ hybridisation of transplants injected with GFP alone, Xcad-11, extracellular (Δe) or cytoplasmic (Δc) deletion mutants. The transplant-containing side (+) is compared with the control side (–) of the same embryo. Arrowheads indicate areas of different marker expression, arrows mark the non-migrating graft.

Xcad-11 constructs altered expression of cranial neural crest markers

As it is clear that Xcad-11 RNA injections alter the migration of transplanted cells, we asked whether the CNC pattern was disturbed. Therefore, we analysed the expression patterns of Xcad-11 and the CNC marker genes *AP-2*, *twist* and *Snail* in embryos 18 hours after grafting. The expression of those markers on the untreated side of each embryo served as a control.

The Xcad-11 pattern resembles the migration behaviour of

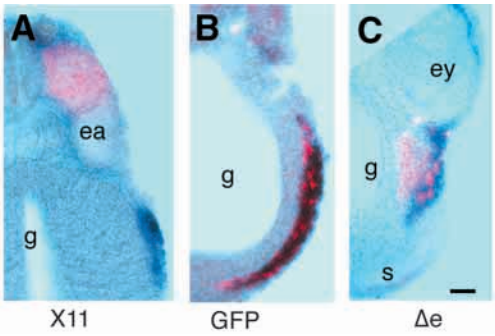

Fig. 3. Localisation of host and donor CNC cells at stage 28. (A) Transverse section showing a Xcad-11-expressing non-migrating transplant (pink), and migrating host CNC cells (blue). (B) Mixture of host (blue) and donor (pink) CNC cells in GFP-RNA injected control embryos. (C) Transverse section showing most of the ΔeXcad-11-expressing, migrating donor CNC cells (pink) separated from the host CNC cells (blue). Pink, immunostaining of Myc-tagged GFP; blue, twist in situ hybridisation. ea, ear vesicle; ey, eye anlage; g, gut. Scale bar: 50 μm.

the grafts (compare Fig. 2A with Fig. 1D). Non-migrating grafts expressing either the full-length or ΔcXcad-11 were identified at the site of implantation (Fig. 2A, arrows), and only few Xcad-11-positive migrating neural crest cells were found. These migrating cells were GFP negative and derived from residual host neural crest. As expected, overexpression of ΔeXcad-11, which resulted in premature migration, led to an increased Xcad-11 signal in the migrating crest streams (Fig. 2A).

Like Xcad-11, *AP-2* expression was also reduced in cephalic crest streams of embryos containing non-migrating transplants (Xcad-11 and ΔcXcad-11, Fig. 2B, red arrowheads). This indicates that a part of the migrating *AP-2* cell population was retained or lost. *AP-2* expression in embryos with ΔeXcad-11 expressing grafts showed no effect compared with the control side (Fig. 2B). Thus, as with Xcad-11, the expression pattern of *AP-2* correlated with the migration behaviour of the grafts.

The analysis of *Snail* expression revealed no differences between the transplant-containing and untreated sides of the embryos, regardless of which Xcad-11 construct was injected (Fig. 2C). We did not, however, observe a reduction of *Snail*-positive cephalic crest streams in embryos containing non-migrating grafts, which would be expected if parts of the host population are removed and replaced by non-migrating donor tissue.

The effect of the different Xcad-11 constructs on *twist* expression was more dramatic than on *AP-2* or *Xcad-11*. *twist* expression was strongly reduced in grafts that expressed ΔeXcad-11 (Fig. 2D, arrowhead), although cell migration was not inhibited by this mutant. In non-migrating ΔcXcad-11 grafts, there was no difference in *twist* expression visible between the grafted and the untreated sides. By contrast, overexpression of the full-length Xcad-11, which contains the β-catenin-binding site, led to reduced *twist* expression (Fig. 2D, arrowhead). Thus, presence of the intracellular Xcad-11 domain led to a reduced *twist* expression on the side containing the transplant.

Taken together, the *AP-2*, *Snail* and *twist* expression patterns of the transplanted embryos were affected by the Xcad-11 constructs in different ways.

Lineage tracing, injection experiments and RT-PCR confirm the transplantation results

Owing to the transplantation procedure, the in situ hybridisation patterns reflect a mixture of host and donor CNC at the transplant-containing side (+ in Fig. 2A-D). However, transverse sections (Fig. 3A-C) allowed the distinction of donor and host CNC as the donor tissue could be identified by immunostaining of the Myc-tagged GFP. As expected, the non-migrating (Xcad-11- or ΔcXcad-11-expressing) grafts were always clearly separated from the migrating neural crest cells. An example for Xcadherin-11-overexpressing donor tissue and twist-expressing, migrating host CNC cells is shown in Fig. 3A. The migrating donor cells, on the other hand, intermingled with host cephalic crest cells (Fig. 3B): cells from a GFP control transplant are twist positive and found among twist-expressing host cells. By contrast, most of the ΔeXcad-11-expressing cells were twist-negative and adjacent to twist-positive host cells (Fig. 3C).

The grafting results were further confirmed by single-sided RNA injections. The RNA of the different Xcad-11 constructs

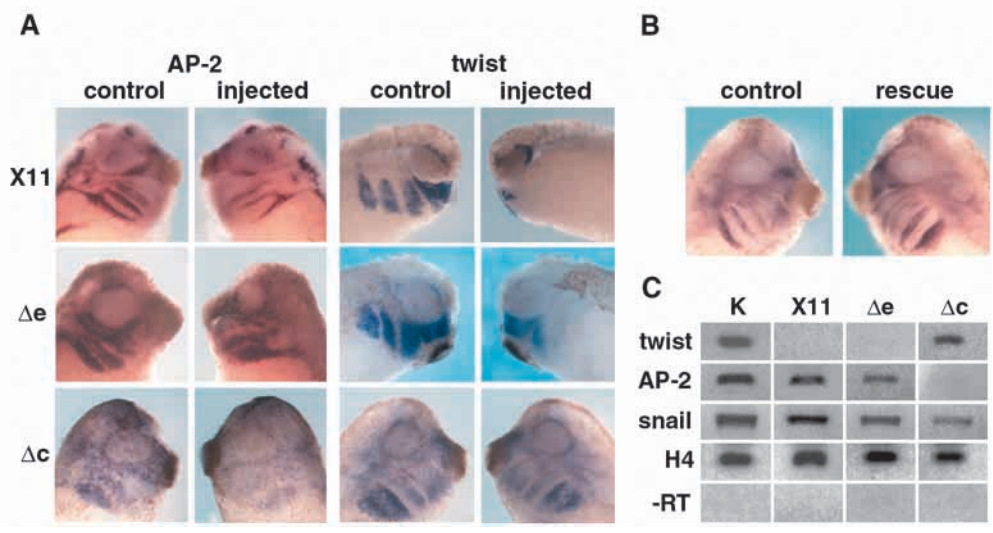

Fig. 4. Xcadherin-11 affects the AP-2 pattern through its adhesion effect, while *twist* expression is influenced by its interference with Wnt/β-catenin signalling. (A) AP-2 and twist in situ hybridisation of embryos injected with 1 ng Xcad-11, 1 ng ΔeXcad-11 or 2.5-ng ΔcXcad-11 RNA in one blastomere at the two-cell stage. (B) *twist* in situ hybridisation of an embryo injected with 1 ng ΔeXcad-11 and 80 pg β-catenin. The injected side (right) is no different from the control side. (C) RT-PCR of half heads of embryos injected as in A. K, control.

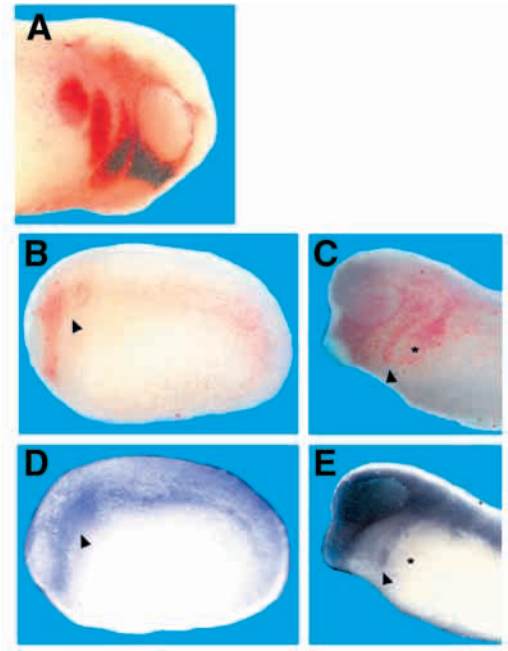

Fig. 5. Double in situ hybridisation. (A) Xcad-11 (blue) and twist (red) at stage 26. (B-E) Double whole-mount in situ hybridisation of stage 20 (B,D), and stage 26 (C,E) embryos for AP-2 (B,C, red) and Snail (D,E, blue).

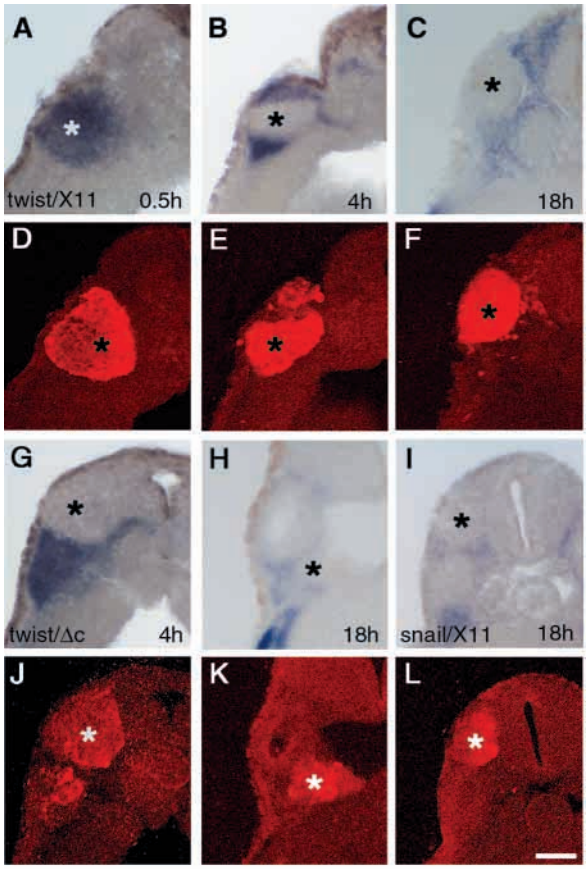

Fig. 6. Inhibition of migration alters neural crest marker expression. (A-C) *twist* in situ hybridisation of grafts expressing Xcad-11 0.5 hours (A), 4 hours (B) and 18 hours (C) after transplantation. GFP-Myc immunostaining of the corresponding sections shown in (D-F). ΔcXcad-11 overexpressing graft 4 hours (G) and 18 hours (H) after transplantation, all stained with twist in situ hybridisation probe. (I) Xcad-11-expressing graft 18 hours after transplantation stained with Snail in situ hybridisation probe. (J-L) GFP-Myc immunostaining of the corresponding sections. Asterisk marks the graft centre. Scale bar: 50 μm.

was co-injected with GFP-RNA into a single blastomere at the two-cell stage, and the embryos were analysed by whole-mount in situ hybridisation at stage 28. In these experiments, not only the CNC, but also the surrounding tissues express the injected cadherin constructs. Nevertheless, we obtained the same results as in the grafting experiments. The *Snail* subpopulation was not affected by the different injected constructs (data not shown). The *AP-2* signal was reduced by wild-type Xcad-11 or ΔcXcad-11, while ΔeXcad-11 had no effect (Fig. 4A). *twist* expression was strongly reduced by ΔeXcad-11 or full-length RNA injection at the injected side (Fig. 4A). In the case of the ΔeXcad-11 construct, *twist* expression was decreased, on average, in 75% of the embryos (87 embryos, five experiments). Most strikingly, endogenous *twist* expression was recovered in 99% of the embryos (88 embryos, three experiments, Fig. 4B) after co-injection of β-catenin. Thus, downregulation of *twist* in migrating, ΔeXcad-11-expressing CNC was most probably caused by β-catenin depletion from canonical Wnt signalling.

We used RT-PCR to validate the effects of our cadherin constructs on neural crest marker gene expression. The

Table 1. Summary of the transplantation experiments

mRNA	mRNA amount	Number of experiments	Number of transplants	Inhibition of migration
GFP	100 pg	16	147	*14%
Xcad11	250 pg	1	4	0%
	0.8 ng	2	25	28%
	1 ng	4	69	34%
	1.6 ng	1	2	100%
ΔeXcad11	250 pg	1	5	0%
	1 ng	6	90	18%
	2.3 ng	1	10	0%
ΔcXcad11	1 ng	1	9	34%
	2.5 ng	2	28	43%
Xcad11 and ΔcXcad11	0.6 ng, 0.8 ng	1	12	42%
Xcad11 and ΔeXcad11	0.8 ng, 0.8 ng	3	46	20%

*Owing to the experimental manipulations, 14% of the GFP controls do not migrate; a comparable percentage was observed with the ΔeXcad-11 mutant.

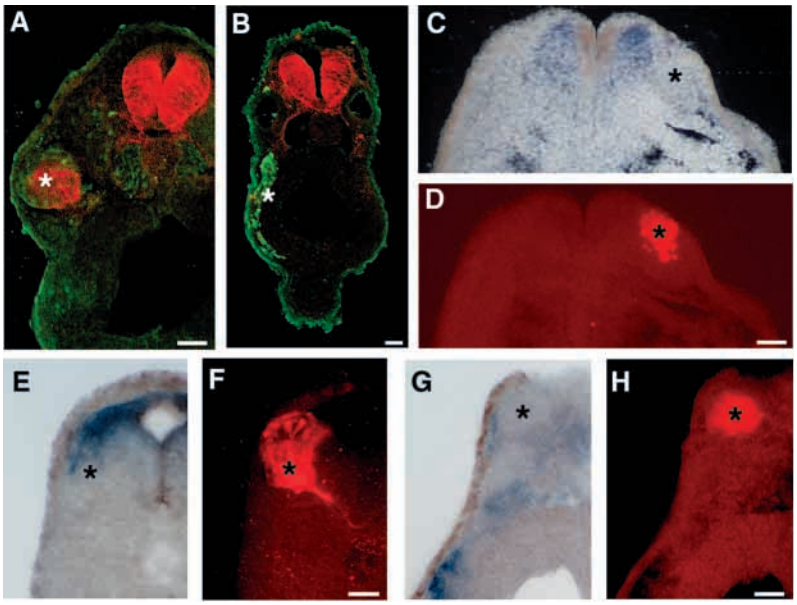

Fig. 7. Homotopic transplants show that non-migrating CNC cells adopt a neural fate. (A) Non-migrating transplant overexpressing ΔcXcad-11 stained with 2G9 antibody, a neural marker. (B) Migrating GFP-Myc-expressing donor CNC cells (green) are negative for 2G9 (red). (C) Whole-mount in situ hybridisation of a ΔcXcad-11-expressing graft shows that sox3 is not expressed 1 hour post grafting. (D) GFP-Myc immunostaining of the section shown in C. (E) In situ hybridisation with nrp-1 probe 18 hours after grafting. Donor embryo was injected with ΔcXcad-11 RNA. (F) GFP-Myc immunostaining of the section shown in E. Upper half of the transplant is nrp-1 positive. (G) In situ hybridisation with twist probe 18 hours after grafting. Donor embryo was co-injected with ΔcXcad-11 and β-catenin RNA. (H) Section shown in G immunostained with 2G9 (red). Donor embryos were injected either with 1 ng Xcad-11, 2.5 ng ΔcXcad-11 or 2.5 ng ΔcXcad-11 plus 80 pg β-catenin RNA. Asterisks mark the graft centres. Scale bars: 50 μm.

expression levels of *AP-2*, *twist* and *Snail* were compared between the RNA-injected and non-injected sides of tadpole heads. Fig. 4C shows a representative RT-PCR of one tadpole head for each injected Xcad-11 construct that was tested for all three neural crest markers. Twist was not detected when full-length and ΔeXcad-11 were overexpressed, while ΔcXcad-11 had no effect on *twist* expression. There was no reduction of AP-2 RNA in Xcad-11- or ΔeXcad-11-injected embryos, while ΔcXcad-11 injection resulted in loss of the AP-2 band. The level of AP-2 RNA in embryos overexpressing ΔcXcad-11 or full-length Xcad-11 varied between undetectable and normal levels in different experiments, while the results of *twist* expression were reproducible. This was most probably due to different molecular effects; interference with Wnt/β-catenin signalling in the case of twist exhibits a stronger phenotype than inhibition of crest migration alone, as seen for AP-2. We also analysed the expression of *Snail* after RNA injections of the different Xcad-11 constructs, but were unable to detect significant alterations in RT-PCR analyses (Fig. 4C). This confirmed the in situ hybridisation results.

In summary, Xcadherin-11 affected the *AP-2*-, *Snail*- and *twist*-expressing CNC domains in different ways: the *twist* subpopulation was diminished, predominantly owing to Xcad-11 interfering with Wnt/β-catenin signalling, while the *AP-2* subpopulation was reduced via cadherin-mediated adhesion. *Snail* expression was not affected in our various experimental systems.

The cranial neural crest consists of heterogeneous cell subpopulations

As the Xcad-11 constructs in transplantation and injection experiments affected CNC marker expression differently, we addressed the question of whether the cephalic crest represents a heterogeneous cell population. To analyse this, double in situ hybridisation was performed. When the Xcad-11 and twist domains were compared directly in the same embryo, *Xcad-11*-expressing cells (Fig. 5A, blue) were found more dorsally located than the *twist*-expressing ones (Fig. 5A, red). The *AP-2* and *Snail* expression patterns were compared by single and double in situ hybridisation from stage 20 up to stage 27. The temporal and spatial expression of both markers differed slightly during CNC migration: at stage 20, the mandibular stream of the AP-2 pattern had already separated from the emigrating hyoid stream (Fig. 5B, arrowhead), while the *Snail* expression was also found between these streams (Fig. 5D). Later, at stage 26, the branchial stream showed expression of *AP-2* (Fig. 5C, asterisk) but not *Snail* (Fig. 5E, asterisk). This indicates that the *Snail* expression domains diverge from the *AP-2* domains. The main differences were observed around stage 26-27, while in later stages, Snail was also present in the branchial stream (see Fig. 2).

Taken together, the expression patterns of Xcad-11, AP-2, twist and Snail only partially overlap, indicating that these markers may temporally form separate CNC subpopulations.

Inhibition of migration abrogates the undifferentiated neural crest state and results in neural differentiation

The next issue to resolve was whether the non-migrating CNC transplants maintained their neural crest character. Therefore, we analysed these transplants in later stages for expression of *twist*, *Snail* and *AP-2*, which are markers for undifferentiated migrating CNC cells. Although a *twist* signal was detected directly after healing in Xcad-11 expressing grafts (Fig. 6A,D), the signal began to fade 4 hours after grafting (Fig. 6B,E) and was completely lost 18 hours after grafting (Fig. 6C,F). This was also observed when ΔcXcad-11 was overexpressed in the grafts (Fig. 6G,H,J,K). As this mutant does not bind β-catenin (Fig. 1B), loss of the twist signal in non-migrating crest cells did not result from inhibition of Wnt/β-catenin signalling. *Snail* expression also decreased in non-migrating transplants expressing either full-length Xcad-11 (Fig. 6I,L) or ΔcXcad-11 (data not shown). Expression was completely lost 18 hours post-grafting (Fig. 6I,L). AP-2 was not suitable for this assay, as it is expressed in specific domains of the brain, making a clear distinction between neural crest cells and neural epithelium impossible.

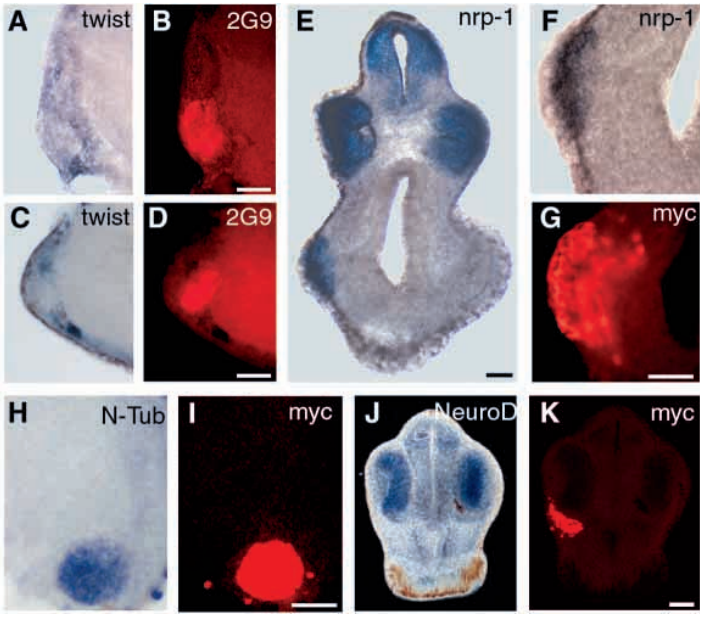

Fig. 8. Non-migrating CNC cells also adopt neural fate after heterotopic transplantation into the pharyngeal pouch area. (A) Xcad-11-expressing transplant was found to be twist negative in whole-mount in situ hybridisation but (B) positive for 2G9 neural marker expression in immunostaining. (C) Transplant from a donor co-injected with ΔcXcad-11 and β-catenin RNA was twist negative in whole-mount in situ hybridisation but (D) positive for 2G9. (E) Transplant from a donor co-injected with ΔcXcad-11 and β-catenin RNA shows nrp-1 expression in whole-mount in situ hybridisation. (F) Higher magnification of the transplant seen in E. (G) GFP-Myc immunostaining of the section shown in F. (H) N-Tubulin-positive transplant from a donor injected with ΔcXcad-11 RNA. (I) GFP-Myc immunostaining of the section shown in H. (J) NeuroD-positive transplant from a donor injected with ΔcXcad-11 RNA. (K) GFP-Myc immunostaining of the section shown in J. Donor embryos were injected either with 1 ng Xcad-11, 2.5 ng ΔcXcad-11 or 2.5 ng ΔcXcad-11 plus 80 pg β-catenin RNA. Scale bars: 50 μm.

The loss of CNC marker expression raised the question of what tissue types differentiate in these non-migrating transplants 18 hours after grafting. Most strikingly, all non-migrating grafts were positive for the neural marker 2G9 (Fig. 7A), which is specific for brain, spinal chord and lateral line (Jones and Woodland, 1989). In migrating cephalic crests streams of GFP controls (Fig. 7B) and ΔeXcad-11-expressing transplants (data not shown), 2G9 staining was not detected. Moreover, the non-migrating transplants became positive for *nrp-1* (Fig. 7E,F). This is a general neural marker (Knecht et al., 1995), which is expressed in the central nervous system (CNS), and transiently in developing cranial ganglia and nerves at stage 20-24, but not in migrating CNC cells from stage 24 onwards.

Because β-catenin signalling is required to maintain *twist* expression in migrating crest cells (Figs 2, 4), we attempted to sustain *twist* expression and block *nrp-1* and 2G9 expression in non-migrating grafts. This was done by co-injection of β-catenin RNA either with full-length or ΔcXcad-11 RNA. As ΔcXcad-11 RNA is unable to bind and deplete β-catenin from the canonical Wnt-signalling pathway, this co-injection experiment would resemble an overexpression of β-catenin. As seen in the case of ΔcXcad-11 (Fig. 7G), *twist* expression could not be rescued by β-catenin and the grafts became positive for the 2G9 marker (Fig. 7H). This was also observed when wild-type Xcad-11 was co-expressed with β-catenin (data not shown). Thus, neuralisation of non-migrating grafts could not be prevented by adding β-catenin.

To rule out the possibility that neural marker expression was caused by contamination of tissue with neural epithelium, we controlled the accuracy of our transplantations. This was done by in situ hybridisation using probes for nrp-1, N-Tubulin, sox2 and sox3 1 hour after graft insertion. As shown for sox3 (Fig. 7C,D), and summarised in Table 2, the transplants were negative for neural markers briefly after grafting. Note that CNC is distinguishable from the neural epithelium at the time of transplantation by its lateral position, translucent appearance and loose packing.

Heterotopic grafting did not prevent neuralisation of non-migrating grafts

The next approach to rescue the neural crest marker expression in non-migrating transplants was heterotopic transplantation. To examine whether the deficit of extrinsic signals, which are normally present along the cranial migratory routes, resulted in the switch to neural marker expression, we transplanted non-migrating grafts into the presumptive pharyngeal pouch area. To our surprise, these non-migrating grafts expressing full-length Xcad-11 (Fig. 8A,B) or ΔcXcad-11 (data not shown) and were positive for the neural marker 2G9, but negative for *twist* expression. Additionally, we tried to rescue *twist* expression by co-injection of β-catenin RNA. As shown in Fig. 8C,D, β-catenin RNA co-injection was unable to sustain *twist* expression. Furthermore, the heterotopic transplants expressed *nrp-1* (Fig. 8E-G). Interestingly, in all these heterotopic transplants 2G9, or *nrp-1* staining was most prominent in the centre of the graft.

Table 2. Expression patterns

Predominant expression in	Probe	1 hour after grafting stage 19	22-24 hours after grafting stage 32
CNS	nrp-1	–/+*	+
	N-Tubulin	–	+
	sox3	–	n.d.
	sox2	–	n.d.
	2G9 (mab)	–	+
PNS	NeuroD	–	–
	Xcadherin-6	n.d.	–
CNC	Twist	+	–
	AP-2	+	–
	Snail	+	–

Donor embryos were injected with 2.5 ngΔcXcad-11 RNA. No differences were observed between homotopic or heterotopic transplantations.

*nrp-1 was found transiently expressed in migrating CNC cells between stage 19-20, which explains why 30% of the analysed transplants were positive for nrp-1 at these stages.

n.d., not determined: the marker is not expressed or its expression is restricted to a small area of the brain.

Non-migrating CNC grafts express CNS-specific neural markers

As neural crest cells contribute to the formation of peripheral nerves and ganglia, we tried to define the neural character of the transplants more closely. The embryos containing grafts were subjected to in situ hybridisation at tailbud stage, using probes for marker genes which are predominantly expressed either in CNS or PNS (Table 2). It is noteworthy that genes exclusively expressed in CNS or PNS are not known in vertebrates. In spite of this limitation, we found that the transplants express neural markers as 2G9, *nrp-1* and *N-Tubulin* (Fig. 8A-I), but not *NeuroD* (Fig. 8J,K) and *Xcadherin-6*, which are most prominent in ganglia and nerves of the PNS (see summary in Table 2).

Xcad-11 and deletion constructs do not affect induction of neural plate and cranial neural crest

Overexpression of cadherin constructs often results in abnormal phenotypes with altered gene expression, owing to interference with Wnt/β-catenin signalling or changes in adhesion. One important question is whether the injected Xcad-11 constructs affect the specification of neural epithelium versus neural crest or the physical segregation of these tissues.

To this end, we analysed the morphology of the neural plate, CNC, placodes and peripheral nerves by in situ hybridisation in embryos that were injected with various Xcad-11 RNAs into one blastomere at two-cell stage. N-CAM and nrp-1 were used as markers for neural plate, twist, AP-2 and snail for CNC, sox2 and sox3 for neural plate and placodes, NeuroD and Xcadherin-6 for PNS. Embryos were co-injected with GFP-RNA. Before the in situ hybridisation, the embryos were selected for proper single-sided GFP distribution. Expression of neural markers was found to be unchanged (Fig. 9A,C,D,H,I). The nerves and ganglia of the PNS were formed normally (Fig. 9F,G). Transverse sections demonstrate the proper localisation of the GFP (Fig. 9I). Importantly, the induction of CNC was not inhibited by expression of ΔcXcad-11 (Fig. 9B), ΔeXcad-11 (Fig. 9E) or full-length Xcad-11 (data not shown). The strongest effect observed was a slight reduction of twist signal on the injected side (Fig. 9E). Thus, the dramatic downregulation of *twist* expression in ΔeXcad-11 and wild-type Xcad-11 RNA injected embryos (see Fig. 5) takes place at a later stage, during CNC migration.

DISCUSSION

As we have already shown the expression of Xcad-11 in migrating CNC cells (Hadeball et al., 1998), this paper focuses on the function in migration and subsequent specification of these cells. Our results provide strong evidence that Xcad-11 restrains neural crest migration. We were able to block or accelerate migration by expressing different cadherin mutants in cephalic crest cells. Additionally, by using these mutants in transplantation assays followed by whole-mount in situ hybridisation and immunostaining, we could also analyse some specification aspects: (1) CNC cells lose their undifferentiated state if migration is prevented and become neural instead; and (2) Wnt/β-catenin signalling is required for continuous *twist* expression during the migratory phase.

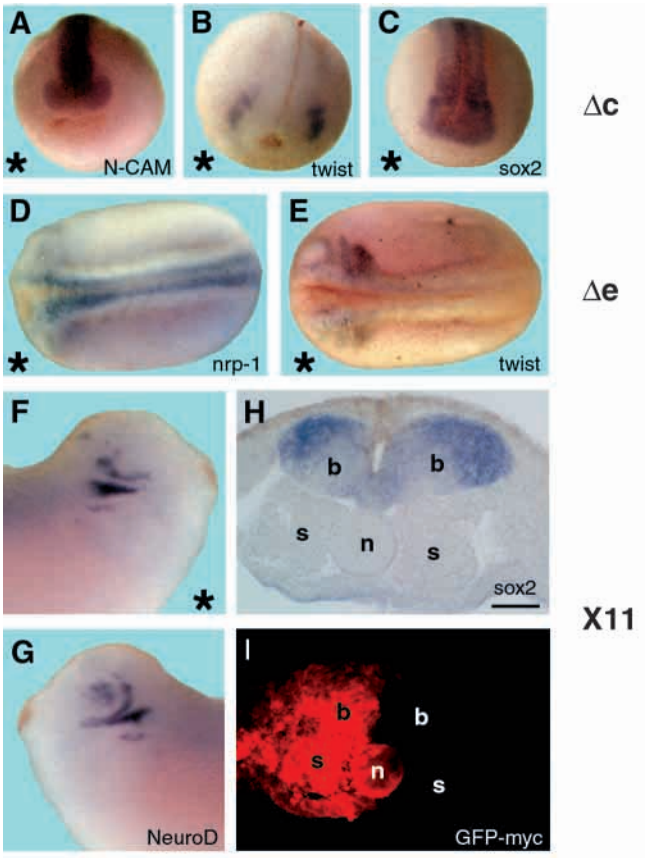

Fig. 9. Expression of Xcadherin-11 constructs neither inhibits neural crest induction nor alters CNS and PNS pattern. (A-C) Stage 15 embryos single-side injected with ΔcXcad-11 RNA. (D,E) Stage 20 embryos injected with ΔeXcad-11 RNA. (F,G) Stage 28 embryo injected with Xcad-11 RNA. (H,I) Transverse sections of stage 28 embryo injected with Xcad-11: (H) in situ hybridisation with sox2 probe; (I) GFP-Myc immunostaining of the same section shown in H. Marker detection as indicated. b, brain; n, notochord; s, somites; asterisk, injected side. Scale bar: 50 μm.

Xcad-11 regulates migration of cranial neural crest cells by its adhesive function

Overexpression of wild-type Xcad-11 and also, surprisingly, its cytoplasmically deleted mutant, led to an increase in cell-cell contacts and inhibition of migration. Although ΔcXcad-11 completely lacks the β-catenin binding site, it acts as a dominant-active mutant like the wild-type cadherin. Therefore, anchorage of Xcad-11 to the cytoskeleton via β-catenin seems to play only a minor role in mediating cell-cell adhesion between neural crest cells. The binding of $p120^{ctn}$ to Xcad-11 also appears not to be necessary for the adhesive function of Xcad-11: based on sequence alignment to classical type I E-cadherin, only 14 amino acids of the juxtamembrane region that interacts with $p120^{ctn}$ (Provost and Rimm, 1999) are preserved in ΔcXcad-11. Furthermore, the conserved core binding sequence (Thoreson et al., 2000) was completely deleted, making $p120^{ctn}$ binding to this mutant unlikely. The results of this work are consistent with previous findings that clustering effects of the transmembrane or extracellular E-cadherin domain could be sufficient to mediate cell adhesion (Ozawa and Kemler, 1998; Huber et al., 1999). In contrast to

ΔcXcad-11, the extracellular deletion mutant (ΔeXcad-11) behaved as a dominant-negative form, and this was demonstrated by premature cell emigration out of the transplant. Interestingly, Nakagawa and Takeichi (Nakagawa and Takeichi, 1998) produced similar results when they introduced N-cadherin deletion mutants into chicken neural crest using an adenoviral expression system. Paralleling our results, they showed that the mutant lacking the β-catenin-binding site partially inhibited migration of melanocyte precursors while the extracellular deletion mutant did not.

Considering Xcadherin-11, our data reveals that the adhesive function of this cadherin is important in regulating the onset and migration velocity of the cephalic crest. This was demonstrated by the enhanced migration of the dominant-negative ΔeXcad-11 mutant, when compared with the GFP control, and by the block of cell migration by dominant-active forms (wild-type Xcad-11, Δc-Xcad-11). As Xcad-11 confers adhesiveness independently of catenin binding in our assay system, an influence of catenins or post-translational modifications of Xcad-11 on the adhesive strength remains elusive. Schneider et al. have reported a potential connection as they observed a reduced catenin expression in migrating neural crest cells (Schneider et al., 1993). Other factors, like small GTPases of the Rho family, seem unlikely to modulate Xcadherin adhesiveness. For example, RhoB, which has been assigned to the delamination process, fades in its expression at the time Xcad-11 is expressed in migrating neural crest cells (Liu and Jessell, 1998).

The question of why migrating cephalic crest cells require adhesive properties still remains. Expression of different cadherins may guide the homing of migrating neural crest cells (Nakagawa and Takeichi, 1995). Proof of this idea remains elusive because of the lack of markers for specific crest derivatives. However, a crude analysis of the cranial skeleton in tadpoles that had been injected with wild-type Xcad-11 and deletion mutants in one blastomere of a two-cell stage embryo, showed no cranial defects on the injected compared with control sides (data not shown). Independent of any effect on the crest derivatives, the typical cranial migration pattern (Sadaghiani and Thiébaud, 1987) remained unaffected. Overexpressing either wild-type Xcad-11 or ΔcXcad-11 at very low doses or ΔeXcad-11 at high doses, all of which show a migratory phenotype, did not disturb the pattern. We conclude from this data that endogenous Xcad-11 reduces the migratory velocity and plays no role in separating the mandibular, hyoid and branchial stream. Thus, Xcadherin-11 function is distinct from that of ephrin receptors, whose dominant-negative expression leads to fusion of crest streams (Helbling et al., 1998).

Xcad-11 function and neural crest specification

The existence of different cell subpopulations with different migratory behaviour has been discussed for *Xsnail*, *Xslug* and *Xtwist* (Linker et al., 2000). Moreover, subpopulations can also exhibit overlapping domains as seen by double in situ hybridisation for twist and Xcad-11 (Fig. 5A), and AP-2 and Snail (Fig. 5D-E). Interestingly, the expression of Xcad-11 and deletion mutants had different effects on the twist, AP-2 and Snail subpopulations, confirming the heterogeneous character of the CNC. One drawback of the assay system used here is that whole-mount in situ hybridisation does not resolve expression profiles of single cells. Therefore, the role of Xcad-11 in segregating cell clusters within one cephalic crest stream will remain elusive until detection of the markers can be improved.

Clustering and restraining CNC cells on their migratory routes probably alters their specification. Although, based on inhibition of BMP and activation of Wnt/β-catenin and/or e/bFGF signalling, the neural crest fate is defined at the premigratory stage (Mayor et al., 1995; LaBonne and Bronner-Fraser, 1998), further specification depends on exposure to extrinsic factors along the migratory routes (Hall and Hörstadius, 1988; LaBonne and Bronner-Fraser, 1999). Some of these molecules have been identified for trunk neural crest using cell cultivation (Le Douarin and Smith, 1988; Anderson et al., 1997), while those important for the differentiation of CNC are still unknown. By preventing CNC cells from migration, we were able to abolish the undifferentiated state of CNC prematurely. This was seen in non-migrating grafts, which started to lose *Snail*, *AP-2* and *twist* expression 4 hours after transplantation. However, this was not caused by blocking Wnt/β-catenin signalling via depletion of β-catenin, because expression of ΔcXcad-11 had the same effect as the full-length form. In addition, β-catenin co-expression in these non-migrating grafts did not prevent fading of the neural crest marker twist, and upregulation of neural markers. This is especially interesting, as the twist subpopulation needs β-catenin to maintain *twist* expression in migrating cells. Loss of *twist* expression in migrating, ΔeXcad-11-expressing CNC cells was rescued by co-expression of β-catenin (Fig. 4B). The unexpected switch from undifferentiated CNC to the neural state in non-migrating grafts can be explained in two different ways: (1) the initial neural crest induction is reversible, and the CNC becomes neural epithelium once again; and (2**)** the neural crest differentiates prematurely into neural crest derivatives, e.g. nerves and ganglia of the PNS. Our extended in situ hybridisation study (Table 2) promotes the idea that the non-migrating grafts differentiate into neural CNS-like tissue rather than PNS-specific structures. In addition, our results demonstrate that increased cell-cell adhesion, which leads to compaction of CNC, induces neural differentiation. As we could not observe the activation of neural marker genes in migrating heterotopic grafts, the adhesion effect seems to be more important than the influence of extrinsic factors. Support for our findings comes from neural crest cell culture studies. It has been shown by Hagedorn et al. (Hagedorn et al., 1999) that clusters of neural crest cells, in contrast to single cells, differentiate into neural cells at the expense of non-neural derivatives, independently of the type or concentration of added differentiation signal (BMP-2, TGFβ). In addition, ganglion formation in neural crest derivatives correlates with upregulation of adhesion molecules (Akitaya and Bronner-Fraser, 1992).

Induction of neural crest is not disturbed by Xcad-11 overexpression

Overexpression of β-catenin or Xwnt-1, Xwnt-8 and Xwnt 7B in combination with noggin or chordin resulted in an increase and expansion of the neural crest markers, while expression of gsk-3β or dnXwnt-8 had the opposite effect (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998). We expected that expression of

Xcad-11 constructs with β-catenin binding sites at the time of neural crest induction should result in the same phenotype, owing to interference with canonical Wnt signalling (Fagotto et al., 1996; Gradl et al., 1999a; Gradl et al., 1999b). This phenotype was not observed. The proteins derived from the injected RNAs were strongly expressed and correctly localised (data not shown). Most likely, gsk-3β overexpression is more efficient in blocking Wnt/β-catenin signalling than depletion of β-catenin by cadherin expression. This, however, would contradict our findings that binding of β-catenin to Xcad-11 is sufficient to repress *twist* expression in the migratory phase. The discrepancies between the former reports and our data may result from the different assay systems. While the induction of neural crest marker genes by canonical Wnt signalling was analysed in animal caps injected with noggin/chordin and Xwnt-1, -3A, or -7 RNA, our analysis focused on the in vivo situation, which might include other putative inducers. Interestingly, blocking canonical Wnt-signalling by gsk-3β expression in the whole embryo did not repress *Krox-20* in the rhombomeres. Instead, the stream of Krox-20-positive neural crest cells was lost (Saint-Jeannet et al., 1997). This could be explained by a late Wnt/β-catenin signalling defect at the migratory stage. Our findings demonstrate that Wnt/β-catenin signalling is also essential for late events in CNC development, in particular to the maintenance of *twist* expression during the period of CNC specification. Late influence of β-catenin, especially on neural crest specification, might also play a role in pigment cell formation. This neural crest subpopulation increased in presence of β-catenin at the expense of neurones and glia cells in zebrafish (Dorsky et al., 1998). Similar observations were made in mice, showing that melanocyte formation was dependent on wnt-1 and wnt-3a signalling (Ikeya et al., 1997; Dunn et al., 2000). Interestingly, in *Xenopus*, components of the canonical Wnt signalling cascade, e.g. Xfz7, XLef-1, XTcf-3, are expressed in migrating cranial neural crest (Molenaar et al., 1998; Wheeler and Hoppler, 1999), stressing a putative function in CNC specification.

Taken together, our data support the model of Mayor et al. in which the decision between neural plate, neural fold and epidermis is made at the premigratory stage (Mayor et al., 1998). Wnt/β-catenin signalling is discussed to contribute to the induction of CNC at premigratory stage. However, in this model the major role of Wnt factors is seen in maintaining the neural crest differentiation program later, at the migratory stage. Our findings confirm this idea, but also supplement the model, as we identified additional factors important for CNC specification: increased cell-cell adhesion and block of migration leads to neural differentiation, while only migrating CNC cells are able to maintain the undifferentiated neural crest state. The migrating CNC cells represent a heterogeneous cell pool with a balanced Xcadherin-11 expression. Xcad-11-mediated adhesion restrains CNC cells, and might allow their prolonged exposure to extrinsic factors. Because Wnt/β-catenin signalling is essential for the *twist*-expressing CNC subpopulation, this signal must belong to the group of extrinsic factors.

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