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Review

Involvement of endothelin receptors in normal and pathological development of neural crest cells

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CONTENTS

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Introduction

Endothelin receptors (Ednr) are members of the family of G protein-coupled receptors (GPCR) with seven transmembrane domains. Their ligands, endothelins (Edn), constitute a family of peptides, all of which are 21 amino acids long. Three members of this family have been described: Edn1, Edn2 and Edn3 (Sakurai *et al.*, 1992). Active endothelin peptides are produced by the proteolysis of larger precursors, known as big endothelins, which consist of 38 amino acids. This crucial step is catalyzed by specific endothelin-converting enzymes (ECE-1 or ECE-2). Three subtypes of Ednr (A,B,C) have been distinguished, on the basis of relative affinities for the various Edns (Sakurai *et al.*, 1992). Ednra has a high affinity for Edn1 and Edn2 and a low affinity for Edn3. Ednrb has similar affinities for all three ligands. Ednrc has a high affinity for Edn3 and a low affinity for Edn1 and Edn2. The cDNAs for these receptors have been obtained and the corresponding genes isolated from the genomes of various vertebrates (see Table 1 for references). The nomenclature of genes and proteins is the one proposed by http://www.informatics.jax.org. The entire cDNA for *Ednra* has been obtained from chicken, Xenopus and various mammals, including mouse and human. Partial *Ednra* cDNAs have been obtained for rabbit and zebrafish. The structure of the *Ednra* gene is entirely known for humans but partially for mice. The complete *Ednrb1* (classically referred to as *Ednrb*) cDNA is available for humans, mouse, rat, pig, cow, sheep and zebrafish, but only partial cDNA have been described for horse, chicken and quail. The structure of the *Ednrb* gene has been fully described in humans and cow, and partially described in zebrafish. A gene encoding another Ednr of the B subtype has been cloned and called *Ednrb2* in quail and chicken. A putative ortholog of *Ednrb2* was recently identified in medaka fish (Akiyama *et al.*, 2002), but no ortholog has been found in mammals. In humans, a gene encoding a putative endothelin receptor B-like protein has been discovered, but the protein does not bind Edn1 or Edn3 and displays only limited similarity to the quail Ednrb2 (Zeng *et al.*, 1997). The only *Ednrc* cDNA isolated to date is that for *Xenopus laevis;* the protein encoded by this cDNA is very similar to the quail *Ednrb2*, 85 % of amino acid residues in the last transmembrane and cytoplasmic domains similar but only 57% similarity over the

Abbreviations used in this paper: Edn, endothelin; Ednr, endothelin receptor; GDNF, glial-derived neurotrophic factor; MSA, migration staging area; NCC, neural crest cell.

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entire length of the protein (Karne *et al.*, 1993). *Xenopus Ednrc* and quail/chicken *Ednrb2* may be orthologous (Parichy *et al.*, 2000). If this is the case, then a major unanswered question emerges: why do mammals have only one *Ednrb* gene? There are two possible answers to this question: (i) the *Ednrb2/Ednrc* gene was probably created by gene duplication during vertebrate evolution, and one copy was lost in the mammalian lineage, (ii) the presence of *Ednrb2/Ednrc* in *Xenopus*, in birds and in Medaka fish results from convergent evolution. Although possible, this is highly unlikely. Finally, diverse splice variants of *Ednra* and *Ednrb* have been described (see for review Davenport, 2002). The function of these splice variants is not yet fully understood. Thus, there is considerable diversity among the Ednr, which may facilitate fine tuning of the processes in which these receptors are involved.

Endothelins and their receptors are principally known for their involvement in the regulation of blood pressure. Endothelins stimulate vasoconstriction through Ednra signaling in smooth muscle cells of blood vessels and stimulate vasodilatation through Ednrb signaling in endothelial cells via NO production. Endothelins initiate also a very broad spectrum of physiological and cellular responses, including proliferation and hypertrophy of vascular smooth muscle cells and cardiac myocytes, bronchoconstriction, stimulation of natriuretic peptide release from atria, inhibition of renin release from renal glomeruli, stimulation of aldosterone release from adrenocortical cells, modulation of neurotransmitter release, and stimulation of astrocyte proliferation (see for review Miyauchi and Masaki, 1999 and Davenport, 2002). The "endothelin/endothelin receptor" system is also involved in an apparently unrelated domain, the development of neural crest cells in the embryo.

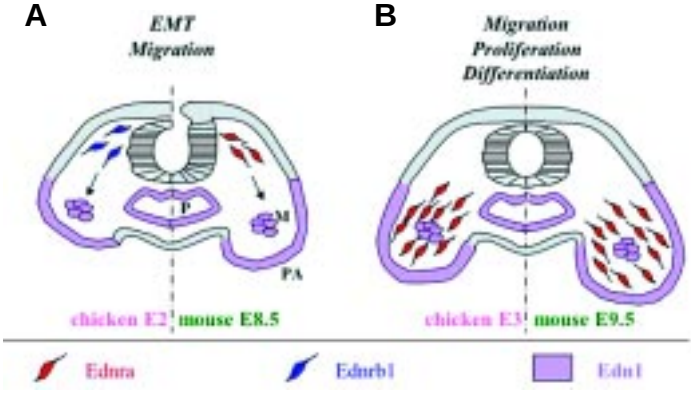

Fig. 1. Expression of Ednra, Ednrb1 and their ligand, Edn1, at the cephalic level of the embryo. (A) *Ednra is expressed in mouse cephalic NCC during their delamination from the neural tube, but is not expressed in chicken NCC (Clouthier* et al.*, 1998; Kempf* et al.*, 1998; Nataf* et al.*, 1998). During the equivalent period, chicken NCC express Ednrb1. The pattern of Ednrb1 production in the heads of mice is not precisely known.* **(B)** *Chicken Ednra expression is activated at around E2.5 in NCC and is maintained in the cranofacial derivatives of these cells and cranial ganglia, whereas Ednrb1 is downregulated. Edn1 is produced in pharyngeal arch ectoderm, pharyngeal endoderm, core paraxial mesoderm and the endothelium of aortic arches in mouse and chicken (Chan* et al.*, 1995; Clouthier* et al.*, 1998; Nataf* et al.*, 1998). EMT, epithelium-mesenchyme transition; M, core paraxial mesoderm; NCC, neural crest cell; P, pharynx; PA, pharyngeal arch.*

Neural crest cells (NCC) are pluripotent cells that arise from the most dorsal part of the neural tube during (in the mouse) or after (in the chicken) its closure. NCC precursors are located at the border between the neural ectoderm (neural plate) and the non neural ectoderm, in the region constituting the apex of the neural fold. NCC emerge after an epithelium to mesenchyme transition (EMT), with the disruption of tight cell-cell contacts with other neuroepithelial cells, the reorganization and redistribution of the cytoskeletal network, and the activation/repression of specific genes (see for review Le Douarin and Kalcheim, 1999).

NCC may be classified into different types, according to position along the antero-posterior axis and the derivatives generated. NCC generated from anterior regions devoid of somites are called cephalic NCC. More posterior NCC are called somitic NCC. These somitic NCC can be further divided into vagal (somites 1-7), truncal (somites 8-27) and sacral (posterior to somite 28) NCC.

Cephalic NCC proliferate actively and migrate throughout the head and pharyngeal arch mesenchyme. These NCC give rise to glial cells and neurons of the cranial peripheral nervous system (PNS). They generate also facial and head dermis, facial muscles, smooth muscles for the walls of the great arteries, most head and facial cartilage and bone (for review see Le Douarin and Kalcheim, 1999). These neural crest (NC) derivatives, which arise from mesoderm in other parts in the embryo, are called ectomesenchyme before their final differentiation.

Somitic NCC proliferate extensively and follow two main migratory pathways: the dorso-ventral and dorso-lateral pathways. The cells that migrate along the dorso-ventral pathway give rise to the non-cranial PNS and to certain endocrine cells, such as the chromaffin cells of the adrenal medulla. The PNS contains the spinal, enteric, sympathetic and parasympathetic ganglia. All these ganglia are composed of neurons and glial cells. Enteric ganglia arise mostly from vagal NCC, but also from sacral NCC. Vagal NCC-derived enteric progenitors enter the foregut mesenchyme migrate from the front to the back of the embryo. Sacral NCC-derived enteric progenitors migrate in from the back to the front of the embryo.

The cells that do not migrate in the dorso-ventral pathway proliferate in a migration staging area (MSA) for several hours and then migrate in the dorso-lateral pathway. These cells give rise to melanocytes. They also give rise to silver iridophores and yellow xanthophores in anamniotes, including teleosts and amphibians. In this case, the situation is further complicated by the change from a larval pigmentation pattern to an adult pigmentation pattern.

A number of fundamental events occurs during normal NCC development: determination of pluripotent neural crests, restriction of the initial pluripotency of NCC, EMT, proliferation, migration, homing and differentiation. A large number of proteins are involved in these processes. For example, the transmembrane Ednr and the Sox10 and Pax3 transcription factors have been shown to be involved in early neural crest cell development. These proteins have been shown to be important for the correct development of neural crest cell lineages (Watanabe *et al.*, 1998; Lang *et al.*, 2000; Potterf *et al.*, 2000; Verastegui *et al.*, 2000).

The role of Edn in neural crest cell development depends on the distribution of Ednr in NCC along the antero-posterior axis in the embryo. In this review, which is divided in three main chapters, we will examine the role of EdnrA in the ecto-mesenchyme, then EdnrB in the enteric ganglia and finally the role of EdnrB in the melanocyte development.

Type-A Endothelin Receptors and Ectomesenchyme

The ectomesenchyme designates the mesenchymal cells derived from the neural crest. These mesenchymal cells are derived from cephalic NCC and from a subpopulation of vagal NCC (somites 1-3). The vagal NCC (somites 1-3) will produce the aorticopulmonary and the conotruncal septa of the heart (Le Douarin and Kalcheim, 1999).

Ednra and Cephalic Development

In mouse, Ednra is first detected at E8.5 in cephalic NCC, just after their emigration from the neural tube, and during their migration towards the pharyngeal arches (Clouthier *et al.*, 1998). In quail, this receptor is detected in NCC from E2.5/E3 onwards, slightly later than the "equivalent" stage in mouse (Kempf *et al.*, 1998; Nataf *et al.*, 1998). Edn1 is produced in the arch ectodermal epithelium, arch core paraxial mesoderm, pharyngeal pouch endoderm and endothelium of pharyngeal arch arteries (Clouthier *et al.*, 1998; Nataf *et al.*, 1998). Thus, the cells of pharyngeal arches may be divided into two populations: the Ednra-positive, Edn1-negative cells, which are ectomesenchymal, and the Ednra-negative, Edn1-positive cells, accounting for all the other cells (Fig. 1).

In birds, Ednrb expression is detected in cephalic NCC before and during EMT, and during cell migration; Ednrb levels then decrease in the ectomesenchyme of the pharyngeal arches (Nataf *et al.*, 1996). Thus, Ednra is the only Ednr present in cephalic NC derivatives.

Ednra has been inactivated in mouse and chicken. In mouse, the *Ednra* gene was disrupted (allele *Ednra*^{tm1Ywa} = *Ednra* -/-) by a classical knockout strategy (Clouthier *et al.*, 1998). In chicken, Ednra was inhibited by specific antagonists *in ovo* (Kempf *et al.*, 1998). Similar results were obtained in these two experiments. The inactivation of *Ednra* results in abnormally small mandibular and hyoid arches. The NC-derived Meckel's cartilage is totally absent, the tongue is small and the development of the middle ear is aberrant, with missing or deformed elements. All abnormalities can be traced to a defect in NCC-derived ectomesenchymal development.

Inactivation of the *Edn1* gene in mice results in a very similar phenotype (Kurihara *et al.*, 1994). In the cranium, inactivation of the *ECE-1* gene results in abnormalities identical to those observed in *Edn1*^{tm1Utj} and *Ednra*^{tm1Ywa} homozygous embryos (Yanagisawa *et al.*, 1998). This genetic evidence demonstrates the importance of the Edn1/Ednra "system" in the correct ontogeny of cephalic tissues.

Disruption of the Edn1/Ednra signaling pathway leads to the malformation or absence of the lower jaw in both mammals and birds, both of which are amniotes. It is therefore of interest to investigate the effects of such a disruption in another gnathostome, such as the zebrafish, which is a teleost. *Sucker* (*suc*), a zebrafish mutant, carries a missense loss-of-function mutation in the *Edn1* ortholog: this mutant has a small lower jaw, and other ventral cartilage structures are also severely affected (Miller *et al.*, 2000). The induction of similar phenotype in amniotes and teleosts by such gene mutations suggests that Edn1/Ednra signaling was involved in cranial NCC development in the common ancestor of amniotes and teleosts.

The cellular events affected by the disruption of Edn1/Ednra signaling may include the migration, proliferation/survival and differentiation of NCC. Studies in the chicken and the mouse have suggested that the migration of cephalic NCC is not affected by the lack of induction or absence of Ednra (Clouthier *et al.*, 1998; Kempf *et al.*, 1998). *Ednra-/-* mouse embryos display significantly less proliferation of NCC on E10.5 than is observed in wild-type embryos. This may account for the abnormally small mandibular and hyoid arches. Moreover, apoptosis is more prevalent after E11.5 in *Ednra-/-* mouse embryos than in wild-type embryos. However, this second cellular event may not necessarily be due to a direct antiapoptotic effect of Ednra, but rather to a secondary effect due to loss of the normal environment and/or differentiation cues of ectomesenchymal cells.

Indeed, disruption of the *Ednra* gene in the mouse affects the expression of genes encoding at least seven transcription factors involved in head development Barx-1, Dlx-2, Dlx-3, Dlx-6, eHAND, goosecoid and hHAND (Clouthier *et al.*, 2000). Gene expression is most severely affected in the distal areas of the pharyngeal arches. These regions subsequently give rise to most of the ventral structures, and these structures are the most strongly affected in *Ednra-/-* embryos. This suggests that Ednra signaling is crucial for certain regionalized populations of NCC. Work with zebrafish *Edn1* mutants has confirmed this finding: Edn1 signaling is required for the ventral expression of dHAND, Dlx-2, Dlx-3, EphA3, goosecoid and msxE, but not for the dorsal expression of Dlx-2 and EphA3 (Miller *et al.*, 2000). Thus, Edn1/Ednra signaling plays a key role in determining the fate of distal/ventral arch NCC.

TABLE 1

SUMMARY OF CLONED EDNR cDNA AND GENOMIC DNA.

Organism / Gene	Teleosts	Xenopus	Gallinaceae	Ungulates	Rodents	Human
Ednra cDNA	AB057355 *Tobita 01'* (Z)	U06633 *Kumar 94'*	AF472618 *Kanzawa 02'* (Ch)	AF416703 *Linden 01'* (Sh)	XM134499 NCBI (M)	L06622 *Elshourbagy 93'*
	AB045356 *Tobita 02'* (Me)			S80652 *Nishimura 95'* (P)	M60786 *Lin 91'* (R)	
				X57765 *Arai 90'* (Co)		
Ednra genomic						D11144-51 *Hosoda 92'*
Ednrb cDNA	AF275636 *Parichy 02'* (Z)		AF472616 *Kanzawa 02'* (Ch)	D90456 *Saito 91'* (Co)	NM007904 *Hosoda 94'* (M)	L06623 *Elshourbagy 93'*
			X99295 *Nataf 96'* (Q)	AF038900 *Santschi 98'* (H)	X57764 *Sakurai 90'* (R)	
Ednrb genomic				D10989-96 *Mizuno 92'* (Co)		D13162-68 *Arai 93'*
Ednrb2 cDNA			Y16089 *Lecoin 98'* (Q)			
			AF472617 *Kanzawa 02'* (Ch)			
Ednrc cDNA		L20299 *Karne 93'*				

For each DNA, the accession number, the first author and the year of publication are given. Endr were cloned from various organisms and species; Ch, chicken; Co, cow; H, horse; Me, medaka; Mo, mouse; P, pig; Q, quail; R, rat; Sh, sheep; Z, zebrafish. The genes which are partially cloned are underlined.

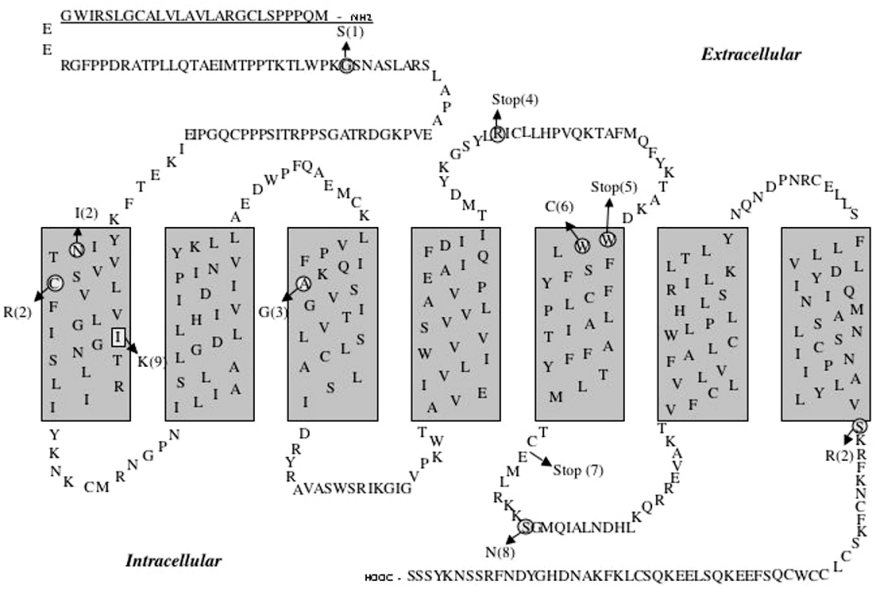

Fig. 2. Ednrb mutations found in human patients with enteric and melanocytic defects. *The secondary structure of human Ednrb is represented. The putative transmembrane alpha-helixes are represented by a grey box. The cleaved peptide signal is underlined. Circles surround a residue which is substitued by another residue, and identified with an arrow in human patients. The square indicates a homologous residue in human and in horse that is substituted in lethal white foal syndrome. References and cases: (1) Familial cases of Hirschsprung disease with coexistence of a RET mutation (Svensson* et al.*, 1998). (2) Mutations in Japanese Hirschsprung disease patients. S390R causes impairement of Edn-induced calcium upregulation and adenylate cyclase inhibition. C109R were not translocated to the plasma membrane (Tanaka* et al.*, 1998). (3) Familial cases of Waardenburg syndrome type IV (Attie* et al.*, 1995). (4) Familial cases of Waardenburg disease type IV. All affected patients were heterozygous for this mutation (Syrris* et al.*, 1999). (5) Isolated case of Hirschsprung disease (Kusafuka* et al.*, 1996). (6) Mutation in Mennonite kindred with many cases of Hirschsprung disesase. Edn-induced calcium signaling is impaired in cells expressing mutated receptors (Puffenberger* et al.*, 1994). (7) Isolated case of Hirschsprung disease. Insertion of a T (nucleotide 878) causes a premature end of translation (Kusafuka* et al.*, 1996). (8) Case of Hirschprung disesase with coexistence of a RET mutation (Auricchio* et al.*, 1999). (9) Lethal white foal syndrome, a congenital anomaly of horses resembling Hirschsprung disease (Metallinos* et al.*, 1998; Yang* et al.*, 1998).*

Some of the transcription factors known to be involved in mouse head development are unaffected: Dlx-1, Hoxa2, MHox, Msx1, and Ufd1 (Clouthier *et al.*, 2000). In the case of the human homolog of *Ufd1, UFD1L,* was found to be deleted in all patients with the CATCH22 phenotype examined (Pizzuti *et al.*, 1997). This phenotype involves abnormal facies and cardiovascular defects and resembles the phenotype of *Ednra -/-* or *Edn1 -/-* mice. Thus, Ufd1 may act in a pathway parallel to Ednra signaling or it may act upstream from Ednra in the same pathway.

It is currently unclear which of the genes with altered expression in *Ednra -/-* or *Edn1 -/-* embryos are directly or indirectly controlled by Ednra signaling. However, a signaling cascade initiated by Ednra has been described (Charite *et al.*, 2001). An enhancer responsible for expression of the gene encoding the transcription factor dHAND in mandibular and hyoid arches was found to respond to endothelin. The Dlx-6 transcription factor binds to this regulatory element. Dlx-6 production is regulated by Ednra signaling, providing a link between this signaling pathway and dHAND gene expression. Other signals must also be involved in the regulation of dHAND production because Dlx-6 is produced in a larger area of the pharyngeal arches than is dHAND. Little is known about the signaling pathways connecting Ednra and Dlx-6. However, $G\alpha_q$ and $G\alpha_{11}$ mutant mice have cranofacial defects (Offermanns *et al.*, 1998), and as these G protein subtypes interact with Ednra, they may mediate the intracellular signaling pathway downstream from Ednra.

Ednra and Cardiac Neural Crest Cell Development

Edn1 is produced in the endothelium of the arch arteries (Clouthier *et al.*, 1998). Subpopulations of NCC in pharyngeal arches 3, 4 and 6 migrate to the cardiac outflow tract. These cells express Ednra and are involved in maturation of the great arteries and of the outflow septation complex. *Ednra-/-* and *Edn1 -/-* embryos have defects in the outflow tracts from the heart, such as interruption of the aorta or extra arteries branching off the carotid arteries. Most mutant embryos have a ventricular septal defect in the heart (Clouthier *et al.*, 1998). Work on chicken has shown that ablation of the cardiac NCC results in a similar phenotype (Creazzo *et al.*, 1998). Thus, the Edn1/Ednra "system" is essential for the correct development of cardiac NCC by means of an endothelium-mesenchyme interaction.

Type-B Endothelin Receptors and Enteric Ganglia

A large body of information is available concerning Ednrb and its ligand, Edn3, before and after determination of the various lineages derived from somitic NCC. This information was gleaned mostly from gene expression studies, and from human and mouse genetics.

Gene Expression

In vertebrates, Ednrb is first expressed at the dorsal tip of the neural tube, in the region in which NCC are present before EMT. This contrasts with Ednra in cranial NCC, which is expressed only after EMT. At the vagal and truncal levels, Ednrb is initially expressed by all NCC, then only by the NCC that migrate along the dorso-ventral pathway and later in most of the derivatives of these cells, such as the adrenomedullary cells and ganglia of the PNS, including enteric ganglia (Nataf *et al.*, 1996). In the gut of quail or chicken, Ednrb expression is restricted to the NCC during the gradual colonization of the entire bowel by these cells (Nataf *et al.*, 1996). No Ednrb has been detected in gut mesenchyme or endoderm. In mouse and zebrafish, Ednrb is expressed by all NCC on both dorso-ventral and dorso-lateral pathways (Parichy *et al.*, 2000). In the gut, contrary to birds, Ednrb is expressed not only in NCC, but also in the gut mesenchyme. It is not expressed in epithelial endoderm cells. The main ligand for Ednrb in the somitic region, Edn3, is produced in gut mesenchyme in both chicken and mouse (Nataf *et al.*, 1998; Leibl *et al.*, 1999).

Genetic Diseases and Mutants

Defects in Edn3/Ednrb signaling and the complete loss of this signaling pathway have been implicated in defects in the development of enteric ganglion cells (Baynash *et al.*, 1994; Hosoda *et al.*, 1994). Mutations in the *Ednrb* and *Edn3* genes have been discovered in zebrafish and in mammals (rat, horse, mouse, and human).

The congenital aganglionosis rat and lethal white foal mutants display a lack of enteric ganglia and melanocytes (McCabe *et al.*, 1990; Gariepy *et al.*, 1996). The *spotting lethal* (= congenital aganglionosis) rat has a 301 bp deletion in *Ednrb* exon-intron 1; this mutation is recessive and leads to an aberrantly spliced non functional Ednrb protein. (Ceccherini *et al.*, 1995; Gariepy *et al.*, 1996; Kunieda *et al.*, 1996). The lethal white foal mutation is a recessive dinucleotide mutation (T353A and C354G) resulting in the replacement of an isoleucine residue by a lysine residue in the predicted first transmembrane domain of the EDNRB protein (Fig. 2) (Metallinos *et al.*, 1998; Yang *et al.*, 1998).

In mouse, the *lethal spotting* and *piebald lethal* loci encode Edn3 and Ednrb, respectively. Various alleles have been isolated or produced for each locus. *Ednrb*$^{s-l}$/*Ednrb*$^{s-l}$ *(piebald lethal),* *Ednrb* ^{tm1Ywa} /*Ednrb* ^{tm1Ywa} *(=Ednrb-/-), Edn3* ^{ls}/ *Edn3* ^{ls} (*lethal spotting*) and *Edn3* ^{tm1Ywa}/*Edn3* ^{tm1Ywa} *(=Edn3-/-)* mice (http://www.informatics.jax.org) have very similar phenotypes: (i) an almost complete loss of pigmentation due to the absence of melanocytes in most of the skin, with the exception of small areas in the head and in the base of the tail, and (ii) aganglionic megacolon due to a lack of enteric ganglia in the distal large intestine. Mild mutations may also be found at these loci in *piebald* mice (Ednrb^{s}/Ednrb^{s}), which manifest white spotting over about 20 % of the coat and a very low penetrance of the mutation as assessed by the enteric ganglia defects it causes. *Edn3* ^{tm1Ywa}/*Edn3* ^{tm1Ywa} mice have more pigment in the head and tail regions than do *Ednrb* ^{tm1Ywa} /*Ednrb* ^{tm1Ywa} mice. Local molecular compensation with other endothelins may occur, accounting for this phenomenon. This compensation is probably active during the early phase of melanocyte development and during the terminal differentiation of melanocytes. Indeed, Edn1 is produced in the skin and in the pharyngeal arches during pigment cell development. As Ednrb binds Edn1 and Edn3 with similar affinity, Edn1 may partially compensate for the lack of Edn3. As expected given the patterns of gene expression observed, no defect in cephalic or cardiac NCC development has been detected in any embryo with mutations in the *Ednrb* or *Edn3* genes. Similarly, Ednra and Edn1 mutant embryos present no defect in coat color or enteric ganglia. Therefore, the "Edn1/Ednra" and "Edn3/Ednrb" systems act independently on two different populations of NCC, separated by their positions along the antero-posterior axis.

In mice with the *Ednrb* ^{sl} and *Ednrb* ^{tm1Ywa} alleles, Ednrb is entirely absent (Hosoda *et al.*, 1994). In *Ednrb* ^{s}/*Ednrb* ^{s} mutant mice, Ednrb levels are 25-30% lower than those in the wild-type, suggesting a mutation affecting regulation of the gene itself (Hosoda *et al.*, 1994). The *Edn3*^{ls} allele contains a missense mutation that prevents conversion of the inactive precursor of Edn3 to its active form (Baynash *et al.*, 1994). The enzyme responsible for the processing of Edn1 and Edn3 is ECE -1. The phenotype of *ECE-1-/-* homozygous embryos resembles that of mutants lacking Edn1 and Edn3: cranial and vascular defects associated with defects in pigmentation and enteric ganglia (Yanagisawa *et al.*, 1998).

Patients with type IV Waardenburg syndrome (= WS4 = Shah-Waardenburg syndrome, [OMIM 277580]) exhibit pigmentation defects, dystopia canthorum, neurosensory deafness and aganglionosis of the colon, resulting in intestinal obstruction and chronic severe constipation. The pigmentation defects result from a lack of melanocytes in the skin and the dystopia canthorum results from a lack of melanocytes in the eyes. The function of melanocytes in the inner ear is not fully solved, but the absence of such cells results in neurosensory deafness. At least, it is known that the presence of melanocytes in the inner ear is required for the integrity of the stria vascularis and the maintenance of the endocochlear potential. Mutations in the *EDNRB* (13q22), *EDN3* (20q13.2-q13.3), and *SOX10* (22q13.1) genes have been implicated in causing WS4 or predisposing patients to this condition (Edery *et al.*, 1996).

Another disease, Hirschsprung syndrome (HSCR) is associated with endothelins and a lack of specific NCC derivatives, which prevents the colon from developing properly. The lack of these cells results in aganglionosis of the colon. Patients with HSCR generally display normal melanocyte development. HSCR has been linked to various genes acting independently or in combination: *EDNRB*, *EDN3*, *ECE1*, *SOX10*, *RET*, *GDNF*, *GRFA1*, *NRTN*, and *ZFHX1B* (Angrist *et al.*, 1998; Pingault *et al.*, 1998; Carrasquillo *et al.*, 2002). Patients heterozygous for *EDNRB* present only one symptom - aganglionosis of the colon (Hirschprung disease *stricto sensu*) - suggesting that the effect of *Ednrb* mutations in humans may depend on the number of copies of the mutated allele. Various

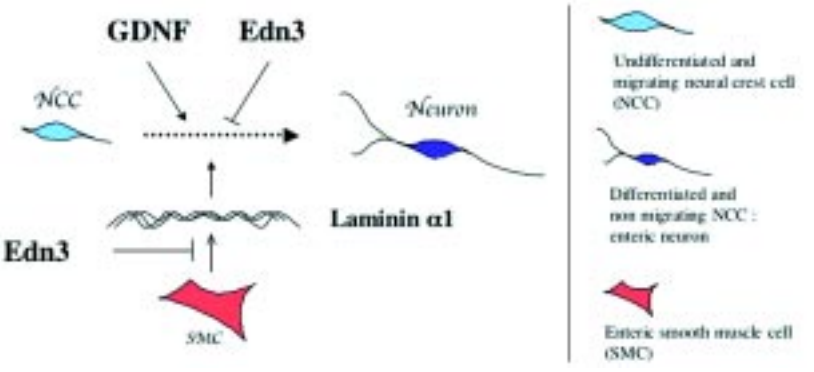

Fig. 3. Differentiation of mouse NCC into neurons is activated by GDNF and laminin alpha 1 and repressed by Edn3. *Edn3 has a direct and an indirect effect on the timing of NCC-derived enteric precursor differentiation. The direct action of Edn3, mediated by Ednrb1 expressed on enteric precursors, counteracts the action of GDNF (Glial-Derived Neurotrophic Factor). GDNF is known to promote both the proliferation and differentiation of enteric precursors (Hearn* et al., *1998). The indirect action of Edn3, mediated by the Ednrb1 expressed on enteric smooth muscle cells, involves inhibition of the synthesis of laminin alpha1 in muscle (Wu* et al., *1999). This extracellular matrix molecule is known to promote neuronal differentiation (Chalazonitis* et al., *1997). Its downregulation should therefore inhibit the differentiation of NCC into neurons.*

mutations have been identified, including a missense mutation at amino acid 276 (tryptophan to cysteine), which impairs the ligand-induced upregulation of cytoplasmic Ca^{2+} concentration (Puffenberger *et al.*, 1994) (Fig. 2). This specific mutation is neither necessary nor sufficient for Hirschsprung disease. The heterozygous missense mutations carried by patients are often inherited from an unaffected carrier (Amiel *et al.*, 1996). This suggests that other loci act as modifiers (Rhim *et al.*, 2000). The SOX10, RET, GDNF, GRFA1, NRTN, and ZFHX1B genes have been identified as possible modifiers, but this list is not exhaustive.

Function of Endothelin 3 in Enteric Ganglion Development

Edn3/Ednrb have various functions depending on the stage of differentiation of the cells. The effects of Edn3 on mouse *in vitro* cultures of enteric NCC isolated from the gut have been investigated: Edn3 inhibits the differentiation of NCC into postmitotic neurons and delays Schwann cell maturation (Hearn *et al.*, 1998; Wu *et al.*, 1999; Brennan *et al.*, 2000). Thus, the interaction of Edn3 with Ednrb present on the surface of the enteric NCC appears to maintain these cells in an undifferentiated state. The proliferation of the precursor cells is not affected by Edn3 *per se:* Edn3 may simply maintain NCC in a state of sensitivity to other factors that have mitogenic effects and/or insensitive to factors that have differentiating effects. Enteric NCC must be in an undifferentiated state if they are to migrate to their final destination in the gut before full differentiation. Indeed, in Edn3-deficient mice, ectopic pelvic ganglia have been observed along the migratory pathway of sacral NCC (Rothman and Gershon, 1984; Payette *et al.*, 1988). These ectopic ganglia may result from the precocious differentiation of sacral NCC that stopped migrating early.

Analysis of *s^l/s^l*<-> wild-type chimeric mice showed that mutant NCC were perfectly able to colonize the distal large intestine (Kapur *et al.*, 1995). This suggests that mutant cells may be helped to migrate by wild-type cells able to respond to Edn. These helper cells may be other NCC or gut mesenchymal cells, both of which express Ednrb in mouse. Nevertheless, it is clear that the terminal aganglionosis observed in *Ednrb* ^{tm1Yma} homozygous embryos is not strictly cell-autonomous, and is probably not NCC-autonomous. Indeed, the *in vitro* induction of Ednrb by Edn3 inhibits the production of laminin alpha-1 and promotes the development of enteric smooth muscle cells which are not NCC derivatives (Wu *et al.*, 1999). Moreover, laminin alpha-1 is known to promote neuronal development *in vitro* (Chalazonitis *et al.*, 1997). Hence, Edn3 may inhibit NCC differentiation by acting both directly on NCC and indirectly on the environment of these cells (Fig. 3). This model is probably limited to mammals as birds express no Ednr in non-neural crest gut mesenchyme. It has been suggested that the initial early growth of avian NCC should generate enough cells for colonization of the entire bowel (Nataf *et al.*, 1996). If this is indeed the case, then the role of Edn3 would be restricted to proliferation and/or survival functions in NCC, indirectly influencing migration.

The phenotype of mice lacking Edn3 or Ednrb differs from that of mice deficient in other molecules known to be involved in enteric ganglion development. RET is a member of the receptor tyrosine kinase superfamily and it transduces signals from GDNF (Glial cell line-Derived Neurotrophic Factor). In *RET -/-* and *GDNF -/-* embryos, vagal and sacral NCC derivatives are uniformly affected along the entire length of the gut, whereas *Edn3-/-* and *Ednrb-/-* embryos are affected only in the large intestine, the hindgut. *In vitro*, GDNF increases the proliferation and neurite differentiation of enteric neural precursors. Edn3 reduces the potential of GDNF to affect cell proliferation and differentiation (Hearn *et al.*, 1998). GDNF may therefore promote the proliferation of migratory enteric precursors and their subsequent differentiation. Edn3 may modulate the action of GDNF, counteracting, in particular, the simulation of differentiation, thereby ensuring that a sufficiently large pool of undifferentiated migratory cells reaches the hindgut (Fig. 3). The absence of GDNF would therefore result in the absence of all enteric neurons because of a general lack of proliferation and differentiation whereas the absence of Edn3 would result in a local lack of neurons. In this case, fully GDNF-stimulated precursors would stop migrating and would differentiate at an early stage of development.

Type-B Endothelin Receptors and Melanocyte Development

The cellular events potentially affected by disruption of the Edn3/Ednrb system include the determination, migration, proliferation/survival and differentiation of melanocyte precursors. Mutations in EdnrB and Edn3 have been discovered in zebrafish and in mammals. The coat color phenotypes are described in the previous part. Under certain circumstances, disruption of this system may also lead to transformation into melanoma.

Gene Expression

In birds, at the vagal and truncal level, the NCC that stay in the MSA decrease their Ednrb1 expression and increase their Ednrb2 expression immediately before migration in the dorso-lateral pathway (Fig. 4). Ednrb2 expression is maintained throughout melanocyte differentiation (Lecoin *et al.*, 1998). In mouse and zebrafish, in which no Ednrb2 has been reported, Ednrb1 is expressed by NCC migrating in both the dorso-ventral and dorso-lateral pathways. Zebrafish Ednrb is expressed in embryonic melanophore, iridophore and xanthophore precursors. It continues to be expressed after melanophore and iridophore differentiation but its expression decreases during xanthophore differentiation (Robert Kelsh, personal communication and Parichy *et al.*, 2000). In chicken, the ligand of Ednrb1 and Ednrb2, Edn3, is produced in the ectoderm and in gut mesenchyme (Nataf *et al.*, 1998). Edn3 is clearly available to NCC migrating along the dorso-lateral pathway. In contrast, at somite stages 17-21 in chicken, Edn3 may be temporarily unavailable to NCC migrating along the dorso-ventral pathway. The diffusion range, and therefore the gradient, of this ligand are unknown.

Development, Ednrb and the Melanocyte Lineage

In *piebald-lethal* mice, the numbers of melanoblasts are very low at E10.5 (Pavan and Tilghman, 1994). Thus, Ednrb signaling is required at least as early as E10.5. Ednrb function has been shown to be required between E10 and E12.5 in experiments based on inducible Ednrb expression and repression (Shin *et al.*, 1999). This period corresponds to the migration of melanoblasts along the dorso-lateral pathway. A partial rescue experiment was performed on mice lacking Ednrb expressing an exogenous Ednrb gene under the control of a melanoblast specific promoter, Tyrp-2 promoter. The expression of Ednrb in already committed melanoblasts was found to rescue coat color in *piebald lethal*/mice (Kos *et*

al., 2002). The Tyrp-2 promoter should be active after determination and after the early proliferation in melanoblasts. Thus, Ednrb seems to be important for the survival, proliferation and/or migration of mouse melanoblasts, but not for their determination or proliferation in the MSA.

In vitro, Edn3 is a very potent mitogen in quail NCC, with a larger number of pigmented cells present in Edn3-treated than in untreated cultures (Lahav *et al.*, 1996). Similar results have been obtained with the mouse (Reid *et al.*, 1996). The interaction of stem cell factor (SCF) with its receptor, Kit, a tyrosine kinase receptor produced by melanoblasts, induces cell proliferation. Interestingly, these two ligands/receptor combinations have an additive, but not synergistic, effect on the proliferation of mouse melanoblasts (Reid *et al.*, 1996). This may suggest that Edn3/Ednrb and SCF/c-Kit act in concert, but via independent signaling mechanisms. This molecular complementation between Ednr and c-Kit signaling was also found in zebrafish. Zebrafish *Ednrb* mutants (*rose*) lack adult LSM (Late Stripe Melanophores), whereas *c-kit* mutants (*sparse*) lack embryonic melanophores and adult ESM (Early Stripe Melanophores) (Parichy *et al.*, 2000; Rawls *et al.*, 2001). Comparison of the patterns of production of SCF and Kit in birds and mammals suggested that these two proteins were required at a later stage in chicken than in mouse. Indeed, in birds, Kit is produced after the beginning of melanoblast migration in the dorso-lateral pathway whereas in mice, Kit is produced in melanoblasts in the MSA (Lecoin *et al.*, 1995; Wehrle-Haller and Weston, 1995; Lahav *et al.*, 1996). It seems likely that, during early melanoblast development in the MSA, Ednrb2 in birds acts similarly to the sum of Ednrb and Kit in mice.

In vitro clonal analyses have shown that the targets for the survival and proliferation activity of Edn3 in the quail are glial (G) and melanocytic (M) unipotent precursors, and common (GM) bipotent precursors if NCC cultures are maintained continuously in Edn3-supplemented media (Lahav *et al.*, 1998). However, when Edn3 induction is transient, it stimulates neuronal (N) and multipotent (NGM) precursors in the short term (Stone *et al.*, 1997).

The ratio of melanocytes (M) to neurons (N) is higher in Edn3-treated cultures than in untreated cultures, but this is not associated with a decrease in the *number* of neuronal precursors present (Lahav *et al.*, 1998). Moreover, the early production of Mitf, a melanoblast-specific transcription factor, in NCC derivatives, is not affected by a null knock-in allele of Ednrb, Ednrb-LacZ, in cultured NCC from mouse embryos (Hou *et al.*, 2002). Therefore, Edn3 probably does not act as an instructive signal reorienting neuronal (N) precursors or directing multipotent cells towards melanocyte (M) differentiation. However, the treatment of clonal *in vitro* quail E7.5 epidermal melanocyte (M) cultures with Edn3 results in the generation of cells expressing glial markers (Dupin *et al.*, 2000). Some cells express both melanocytic and glial markers. Thus, in these conditions, Edn3 seems to be able to dedifferentiate melanocytes and to drive their differentiation into glial cells; Edn3 may therefore be interpreted as providing an instructive signal. It is not clear to what extent the intermediate cells (cells that are not longer melanocytes, but have not yet finished their differentiation into glial cells) are similar to the bipotent glial/melanocytic (GM) precursors observed during normal neural crest development, the proliferation of which is stimulated by Edn3. The relevance of such transdifferentiation *in vivo* is currently unclear.

In quail, successive expression of the two types of Ednrb in NCC is responsible for the pattern of cell proliferation and differentiation. The treatment of NCC cultures *in vitro* with Edn3 seems to result in the downregulation of Ednrb expression and the upregulation of Ednrb2 expression (Lahav *et al.*, 1998). The initial stimulation of proliferation in glial/melanocytic (GM) precursors appears to be mediated by Ednrb and then by both receptors; the transient coexpression of Ednrb and Ednrb2 has been detected *in vitro* in a few cells (Lahav *et al.*, 1998). The later stimulation of proliferation and differentiation in melanoblasts is then mediated by Ednrb2 alone. Given that Edn3 is synthesized by the ectoderm *in vivo*, these data suggest that (i) the initial proliferation of multipotent NCC is mediated by Ednrb, (ii) prolonged exposure to Edn3 results in commitment of these NCC to the melanocyte lineage by repression of Ednrb gene expression and an increase in Ednrb2 levels and (iii) Ednrb2 expression is associated with the proliferation and migration of melanoblasts. It is likely that the specific expression of Ednrb or Ednrb2 in these cells is associated with the transduction of specific signals. The transduction pathways activated by Ednrb and Ednrb2 in NCC are

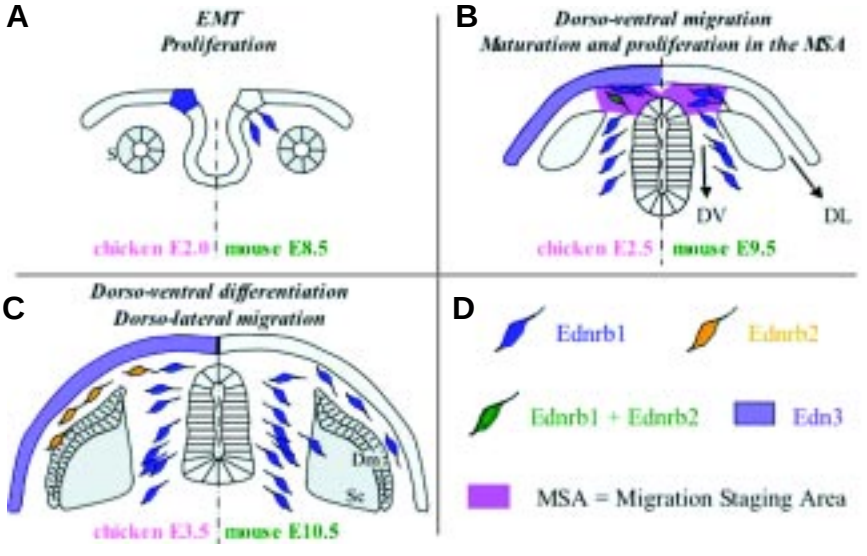

Fig. 4. Expression of Ednrb1, Ednrb2 and their ligand, Edn3, at the truncal level of the embryo. *In birds, Ednrb1 is expressed from E2 onwards in the presumptive NCC in the neural fold. Ednrb1 expression is maintained in NCC migrating in the dorso-ventral pathway (Nataf* et al., *1996). In NCC migrating in the dorso-lateral pathway, Ednrb1 expression in the MSA is downregulated whereas Ednrb2 expression is upregulated (Lecoin* et al., *1998). The simultaneous expression of Ednrb1 and Ednrb2 in individual cells in the MSA has not been demonstrated* in vivo *but has been observed in NCC cultures* in vitro *(Lahav* et al.*, 1998). In the mouse, Ednrb1 is expressed in all NCC, whether these cells migrate in the dorso-ventral or the dorso-lateral pathway. The ligand, Edn3, is produced in the ectoderm and in gut mesenchyme in chicken (Nataf* et al., *1998). Although it is probably also produced in mouse ectoderm at this stage, such production has not been demonstrated experimentally. Dm, dermomyotome; MSA, migration staging area; NCC, neural crest cell; S, somite; Sc, sclerotome; DV, dorso-ventral; DL, dorso-lateral.*

unknown. However, we may believe that Ednrb act upstream of Sox10 because the WS4 phenotype can result from mutations in the *Edn3, Ednrb* or *SOX10* gene. A very limited number of direct information concerning the transduction of Ednrb in NCC derivatives are available. For instance, it was shown that *in vitro* with human melanocytes, which possess only Ednrb (Yohn *et al.*, 1994). In these cells, *Edn3* stimulation increases inositol 1,4,5-triphosphate and intracytoplasmic calcium concentrations. This suggests that a mechanism involving the Gq pathway is linked to the diacylglycerol/inositoltriphosphate pathway (Kang *et al.*, 1998).

In zebrafish, a premature stop codon has been identified in the sequence of the orthologous *Ednrb* gene in the *rose* mutant (Parichy *et al.*, 2000). Ednrb does not seem to be involved in embryonic pigment cell development as the larval pigmentation pattern is normal in the *rose* mutant. In contrast, the adult pattern is abnormal, with *rose* mutants possessing only half as many melanocytes as wild-type zebrafish. This pigmentation pattern results from a failure of the embryo to produce a subset of adult melanocytes, the LSM. The *rose* mutant also displays a severe deficit in the number of iridophores. This mutation has no clear effect on the digestive tract. In evolutionary terms, the differences in the Ednrb requirements of zebrafish and amniotes may be a consequence of a divergence in the function of Ednrb between these taxa and/or duplication of the genome of the zebrafish. Genome duplication has resulted in duplication of all the genes, so one or more additional endothelin receptors, encoded by a potential *Ednrb2* gene, for example, may compensate for the absence of functional Ednrb.

In *Xenopus*, endothelin signaling is involved in pigment dispersion along the microtubule network, within melanophores. This effect is mediated by Ednrc, which is very similar to the Ednrb2 of quail, especially in the C-terminal part of the protein (Karne *et al.*, 1993).

Transformation, Ednrb and the Melanocyte Lineage

Endothelin receptors have also been implicated in the transformation of melanocytes to give metastatic melanomas. The involvement of Ednrb in this process is not surprising, given that tumor progression is often associated with the reactivation of various embryonic genes, especially after loss of differentiation. All human melanoma cell lines tested express Ednrb (Yohn *et al.*, 1994; Zhang *et al.*, 1998; Demunter *et al.*, 2001). Edn1, by binding to Ednrb, promotes the proliferation of cultured melanoma cells and has an anti-apoptotic effect (Eberle *et al.*, 2002). BQ788, a specific antagonist of Ednrb, reduces melanoma cell growth, stimulates various aspects of differentiation, such as enhanced pigmentation and dendricity, and may cause apoptosis in human melanoma cell lines (Lahav *et al.*, 1999). In the nude mouse model, this antagonist affects the growth of human melanoma tumors (Lahav *et al.*, 1999). Thus, Ednrb seems to be crucial for melanoma growth, possibly linked to its role in increasing proliferation during NCC development. In addition, the binding of Edn1 to Ednrb promotes chemokinesis in cultured melanoma cells, and Ednrb antagonists reduce metastasis (Yohn *et al.*, 1994; Lahav *et al.*, 1999). As endothelial cells produce Edn1 and secrete it in a basal direction, it is possible that, *in vivo,* endothelial Edn1 acts on melanoma cells located in the dermis by increasing their mobility, thereby increasing their invasiveness and metastatic potential. E-cadherin may act as a tumor–suppressor in melanoma cells (Pla *et al.*, 2001). It has been shown that Edn1, interacting with Ednrb1, downregulates E-cadherin in human melanocytes and melanoma cells (Jamal and Schneider, 2002). This downregulation may provide a molecular explanation for the effect of Ednrb1 pathways on the invasiveness of melanoma. The enhancement of tumor growth and progression linked to the activation of Ednrb1 is, however, not a general case. In uveal melanomas, the most common cancer in the eye, a reduced expression of Ednrb1 is correlated with a more severe metastatic behaviour of these cells (Smith *et al.*, 2002).

Ednra is not produced by melanocyte precursors, but it has nonetheless been implicated in melanoma formation (Yohn *et al.*, 1994; Ohtani *et al.*, 1997; Lahav *et al.*, 1999). BQ123, a specific antagonist of Ednra, slightly increases the survival of various human melanoma cell lines, simultaneously increasing the production of Ednra itself, resulting in the differentiation of A375 melanoma cells (Ohtani *et al.*, 1997; Lahav *et al.*, 1999). In several human melanoma cell lines and in melanoma tissue, the majority of Ednra transcripts lack exons 3 and 4, and the protein they encode binds Edn with a very low affinity (Zhang *et al.*, 1998). These observations suggest that Ednra inhibits tumor growth and that, except in uveal melanomas, Ednrb stimulates tumour growth.

Conclusion / Perspectives

In adults, dysfunctional endothelin receptor signaling mostly results in cardiovascular defects, independently of neural crest cell derivative defects. In embryos, most of the defects observed affect NCC derivatives. However, some of these NCC derivatives are involved in the development and function of the heart and major vessels.

The anatomic environments of the cephalic and truncal NCC differ. In particular, the cephalic part of the embryo lacks somites. In the truncal part of the embryo, the NCC can migrate between the somite and the neural tube, and between the somite and the ectoderm. The cellular and molecular environments of cephalic and truncal NCC are completely different. In addition, the expression of different receptors on the surface of NCC leads to diversity and to the differential regulation of cell signaling. In particular, the presence or absence of Ednra and Ednrb on the surface of NCC may result in different patterns of NCC behavior. The specific functions of these two receptors could be investigated by studying Ednrb expression under Ednra regulation, and vice versa.

Although the various Ednr play different roles in different NC-derived lineages, they share certain common features. All somitic NCC derivatives express Ednrb in mouse. In mutants lacking this receptor, the derivatives most strongly affected are those that migrate the most: melanocytes and enteric precursors. These cells have to colonize an extremely large area — the entire skin for the melanocyte precursors and the entire gut for enteric precursors. Melanoblast migration requires widespread superficial migration towards the ventral part of the embryo. The migration of enteric NCC involves a ventral and then an antero-posterior or postero-anterior migration. The cells giving rise to the dorsal root ganglia (DRG), parasympathetic and sympathetic ganglia do not migrate to the same extent and are not affected by the absence of Ednrb.

NCC proliferate extensively during their migration, resulting in considerable dispersion. Possible defects in proliferation or survival may have a greater effect in dispersed cells (melanoblast or enteric NCC) than in concentrated cells (DRG, parasympathetic and sympathetic NCC). The phenotype of the concentrated cells may also be less apparent than that of dispersed cells due to a potential community effect. The effects of the defect in dispersed cells may resemble a defect in migration.

In mouse, Ednrb seems to play a role, albeit indirect, in the migration of both enteric and melanocyte lineages. In the gut, the activation of Ednrb appears to prevent NCC from differentiating before they reach their final destination in the hindgut. This receptor therefore plays an indirect role in migration. In melanocyte development, the time-window during which Ednrb is required is clearly related to melanoblast migration; however, the possible direct role of this receptor in migration has not been investigated.

Future work should unravel the intracellular signaling pathways activated by ligand binding to Ednra, Ednrb and Ednrb2 in NCC. Studies *in vitro* with cultures of NCC and their derivatives are likely to prove valuable. Analysis of mutant forms of EdnR in patients has also provided useful insights. For example, the missense W276C mutation of Ednrb1 present in some patients with Hirschprung disease impairs the Gq coupling of the receptor, but not Gi or Go coupling. This suggests that Gq signaling, which involves diacylglycerol, inositol triphosphate and calcium messengers, is involved in enteric glia development (Imamura *et al*., 2000). Studies on cells of the melanocytic lineage should determine whether the signaling pathways activated by Ednr during melanocyte development are completely different from, or have some similarity to the signaling pathways activated during the development of enteric glia.

Summary

Endothelin receptors (Ednr) are G-protein-coupled receptors with seven membrane-spanning domains and are involved in various physiological processes in adults. We review here the function of these receptors during the development and transformation of the neural crest cell-specific lineage. Neural crest cells (NCC) may be classified according to their location in the body. In particular, there are clear differences between the neural crest cells arising from the cephalic part of the embryo and those arising from the vagal and truncal part. The development of cranial and cardiac NCC requires the endothelin-1/Ednra system to be fully functional whereas the development of more posterior NCC requires full functionality of the endothelin-3/Ednrb system. Mutations have been found in the genes corresponding to these systems in mammals. These mutations principally impair pigmentation and enteric ganglia development. The precise patterns of expression of these receptors and their ligands have been determined in avian and mammalian models. Data obtained *in vitro* and *in vivo* have provided insight into the roles of these proteins in cell proliferation, migration, differentiation and transformation.

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