THE HINDBRAIN NEURAL CREST AND THE DEVELOPMENT OF THE ENTERIC NERVOUS SYSTEM

DE NEURAALLIJST VAN DE ACHTERHERSENEN EN DE ONTWIKKELING VAN DE DARMINNERVATIE

PROEFSCHRIFT

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"We respond to fame's trumpeting of an individual scientist, but are often deaf to her orchestrations; yet of all human activities the occupation of science is more like a symphony than a solo".

W. Grey Walter

Voor mijn ouders
Voor Wil

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List of abbreviations

CNS:	Central Nervous System
DiI:	1,1-dioctadecyl-3,3,3',3'-tetramethylincarbo-cyanine perchlorate
E..:	Embryonic Day
ENS:	Enteric Nervous System
HNK-1:	Human Natural Killer cell (monoclonal antibody)
HSCR:	Hirschsprung's disease
ls:	Lethal spotted
RA:	Retinoic Acid
S:	Somite
Sl:	Piebald Lethal

GENERAL INTRODUCTION AND SCOPE OF THIS THESIS

The wonder of things is the beginning of knowledge, as was already stated by Aristotle, the first embryologist known to history. Embryology has remained a source of wonder ever since. It all starts with the fusion of the female egg and the male sperm. Sperm cells were first described by Antonie van Leeuwenhoek (1632-1723) in 1678, who believed them to be parasitic animals present in the male semen, that had nothing to do with reproduction. Nicolas Hartsoeker (1656-1725), the other discoverer of sperm believed that the entire embryonic individual lay preformed within the head of the sperm, as depicted in his famous homunculus (Fig. 1). The first evidence for the existence of the female egg was presented by Reinier de Graaf (1641-1673), although the egg itself was only described in 1827 by Karl von Baer (1792-1876). The actual fertilization process was observed only a century ago by Herman Fol, a Swiss zoologist.

The development of one single cell (the zygote) into a complex organism consisting of many different cell-types and organs, entails a number of processes including cell migration, proliferation, differentiation, growth and pattern formation. During the cleavage stage, the zygote divides a number of times leading to the formation of a blastula. This blastula is then transformed, due to a series of complex cell movements, into a gastrula, a three-layered embryo consisting of endo-, meso- and ectoderm, in which the blue print for segmentation is laid out. Also at this stage, the primitive gut is formed. During neurulation, the mesoderm induces part of the overlying ectoderm to form a neural plate, which subsequently develops into the neural tube. During this process, the vertebrate neural crest arises on the dorsal aspect of the neural tube (Fig. 2). The neural crest is a migratory population of cells, which was first described in chicken embryos by Wilhelm His (1831-1904) in 1868. He described it as a band of particular material, which he called the Zwischenstrang, lying between the presumptive epidermis ('Hornblatt') and the neural tube. Neural crest cells emigrate from the neural tube, either prior to fusion of the neural folds (amphibia and mammals), or shortly following its closure (birds). After initiation of migration, neural crest cells embark upon migratory pathways, characteristic of their axial level of origin,

Figure 1: The human infant preformed in the sperm: Nicolas Hartsoeker's homunculus. (From N. Hartsoeker, 1694, Essai de Dioprique.)

and give rise to a large variety of derivatives. These include ganglionic derivatives, such as the neurons and supportive cells of the peripheral nervous system, ectomesenchymal derivatives, such as the bony and cartilaginous structures of the head, and adrenomedullary and pigment cells (Table 1). Although a great deal is already known about the migratory pathways and range of potential derivatives, there are still many open questions concerning neural crest cell migration and differentiation.

The hindbrain or rhombencephalic neural crest gives rise to the neurons and supportive cells of the enteric nervous system (ENS) (Yntema and Hammond, 1954; LeDouarin, 1982). This local nervous apparatus is embedded in the wall of the gut and is responsible for peristaltic contractions. These are true coordinated reflexes consisting of proximal contractions and distal dilatations, resulting in a craniocaudally directed movement of the contents of the gut. The ENS is considered to be a separate division of the autonomic nervous system (Langley, 1921), and differs from the other components of the peripheral nervous system, i.e. the sympathetic and the parasympathetic ganglia, in that it can mediate reflex activity independently of the central nervous system (CNS) both in vivo (Bayliss and Starling, 1899; Bayliss and Starling, 1900) and in vitro (Trendelenburg, 1917). This indicates that the ENS is a self-contained nervous system, the only such system in the periphery.

Although the gut can function independently from the CNS, it does not normally do so (Roman and Gonella, 1981). Both sympathetic and parasympathetic nerves innervate the bowel (Kuntz, 1963). In addition, sensory neurons located in the vagal and dorsal root ganglia project to the gut (Ewart, 1985). Thus, peristaltic activity can be influenced by the CNS in the normal control of gastro-intestinal function. Apart from its role

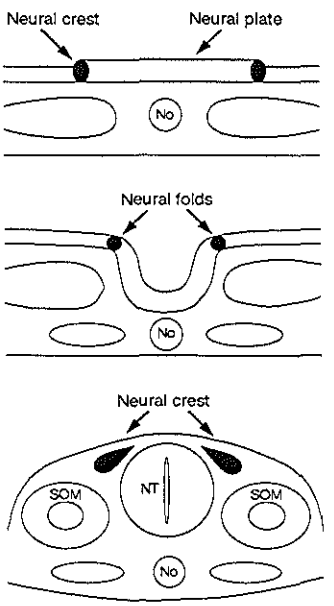

Figure 2: *Schematic diagram illustrating transverse sections through embryos at progressive stages of neural tube closure. The neural folds form from the neural plate under the influence of the notochord (No). The neural folds come together and close to form the cylindrical neural tube (NT). Neural crest cells emerge from the dorsal neural tube and migrate extensively. Som=somite. (Adapted from Selleck et al., 1993)*

Table 1: Derivatives of the different regions of the neural crest

Neural crest region	Mesencephalic	Anterior Rhombencephalic	Posterior Rhombencephalic	Truncal
Pharyngeal arches / somites		1 and 2	3, 4 and 6 / from MO-S5*	Caudal to somite 5
Ectomesenchymal derivatives				
Skeletal	Nasal, orbitary skeleton Trabeculae Sphenoid capsule Cranial vault Anterior squamosal bone Frontal bone Rostrum of the parasphenoid Pterygoid	Reichert's cartilage Parietal bones Palatine, Maxilla Skeleton of the lower jaw (including Meckel's cartilage)	Hyoid cartilage Laryngeal cartilages Otic capsule	
connective / muscle	Meninges of the forebrain Muscles and connective tissue of the face	Tooth buds Muscles and connective tissues of the head and neck region	Mesenchymal components of aortic arch arteries, aorto-pulmonary septum, semilunar valves, thymus, thyroid, and parathyroids	
Ganglionic derivatives (neurons and glial cells)	Cranial nerves I-IV	Trigeminal (V) root ganglion Facial (VII) root ganglion	Glossopharyngeal (IX) root ganglion Vagal (X) root ganglion Cardiac ganglia Enteric ganglia	Dorsal root ganglia Sympathetic ganglia
Other derivatives	Pigment cells	Pigment cells	Pigment cells Carotid body type I cells Calcitonin-producing cells	Pigment cells Adrenal medulla

* from the level of the mid-otic vesicle down to the caudal boundary of somite 5

in intestinal motility, the ENS is also involved in controlling gastrointestinal immunity, the resorption and secretion process, and intestinal blood-flow (Gershon, 1981; Cooke, 1986; Felten et al., 1988).

Scope of this thesis

Knowledge on the cellular and molecular mechanisms of the development of the hindbrain and its neural crest is rapidly increasing, but still far from complete. The neural crest of the posterior rhombencephalon (rhombomeres 6-8) contributes to a number of derivatives, partly ectomesenchymal, such as cells in the outflow tract of the heart (in the media of the aortic arch arteries, the aorto-pulmonary septum and the semilunar valves) and the mesenchymal components of the thymus and parathyroids, partly ganglionic, such as the nervous system of the digestive tract and the cardiac ganglia. The aim of the experimental work described in this thesis is to extend our understanding of the cellular and molecular mechanisms of the development of the ENS. In order to identify and characterize the neural crest cells involved, segments of hindbrain neural crest were ablated in chicken embryos and the effects on ENS development studied. A specific segment adjacent to somites 3-5 was further characterized in in vitro cultures and in an in vivo colonization assay. To identify homing and/or differentiation signals for neural crest cells, the enteric microenvironment of aneural and neural gut was compared. A brief overview of the neural crest and the ENS in various species is given. Finally, the current data on the cellular and molecular mechanisms of ENS development are discussed.

Chapter 1

Introduction to the neural crest

1.1. The evolutionary origin of the neural crest

The evolutionary appearance of the neural crest and of the epidermal neurogenic placodes (local thickenings of the ectoderm, which also contribute to the vertebrate nervous system) has been closely tied to the development of the brain, skull, sensory apparatus and muscular pharynx of the vertebrate head (Gans and Northcutt, 1983). Maisey (1986) stated that the neural crest is the quintessential characteristic of vertebrates: the craniate features resulting from the presence of the neural crest, both directly, through the multitude of tissues produced by the neural crest and indirectly, through the role of the neural crest as an inducer of tissues arising from other germ layers.

The transition from prevertebrates to vertebrates is considered not to be a macroevolutionary change, but rather to represent a series of gradual changes (Gans and Northcutt, 1983; Northcutt and Gans, 1983). The deuterostome invertebrate was a small, filter-feeding, marine organism, who used multiple ciliary bands to drive propulsion, filtration and food transport. It had no specialized sense organs or brain, but a distributed epidermal nerve plexus, coordinating motor response on the basis of local sensory input. Transition to protochordates entailed the development of the notochord, flanked by muscles capable of undulating the notochord, resulting in more effective locomotion. This reduced the need for a ciliated surface, which led to redundancy of part of the sensory capacity and thus of part of the epidermal nerve plexus. This partly redundant epidermal nerve plexus might have evolved into the neural crest in vertebrates.

The shift from passive drift toward powered and oriented movement presumably established the selective advantage for an improvement of gas exchange, which until then had depended solely on diffusion. In agnatha, the first (jawless) vertebrates, this led to the development of a branchial arch system, through which water containing both oxygen and food particles was pumped. This branchial arch system was supported by cartilage, which was the first discrete neural crest tissue in ontogeny that arose from transformation of the epidermal nerve plexus (Maisey, 1986; Hall, 1992).

Modification of the first pair of branchial arches into jaws in gnathostomes, was an important step in the transition from filter-feeders to predators. Prey detection improved with the development of electroreceptors, associated with dentine and enamel, the first hard tissues that derived from the neural crest. In order to further stabilize these new signal sources, secondary specializations developed from the neural crest in the form of bone (and perhaps also cartilage), with the sense organs resting in or among bony plates (capsules). Thus, the neural crest, which was initially a sensory tissue, now also became involved in skeletogenesis. After the development of lungs, which accompanied a terrestrial life style, the branchial arch system evolved into the more complicated pharyngeal arch system found in higher vertebrates.

The first evidence presaging the neural crest as the origin of vertebrate pigment cells

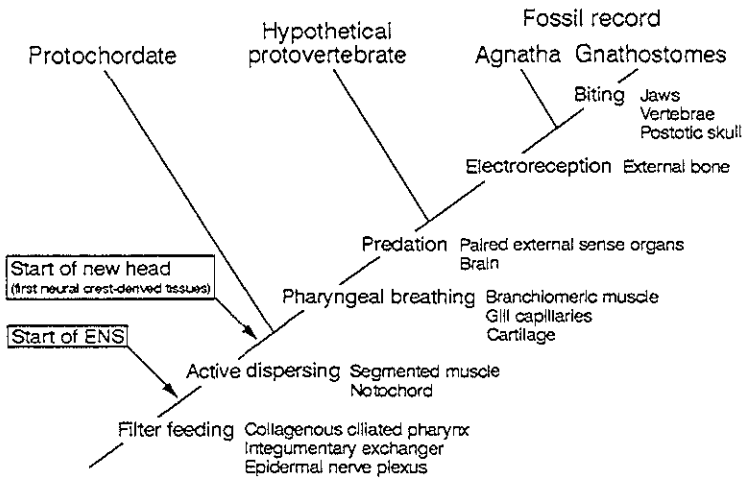

Figure 3: Hypothesized structural and functional transitions in vertebrate evolution. The postulated functional states (capitals) precede the modified structures (lower case letters) involved with them. (Adapted from Gans and Northcutt, 1983)

comes from Tunicates (subphylum Urochordata), who posses a line of pigment cells as a band along the dorsal aspect of the neural tube. These cells are the first and only cells in Tunicates, which are not fully determined and can switch to the muscle cell phenotype, giving further indications that these cells represent derivatives of protoneural crest cells. This shows that all neural crest-derived tissues, characteristic for vertebrates, developed more or less independently in various protochordate and early vertebrate species (Fig. 3).

1.2. The neural crest in amphibians

At the closure of the neural folds, amphibian neural crest material, lying in the ridges on each side, fuses together in the midline and constitutes a wedge-shaped cell-mass, which separates the two halves of the neural tube dorsally. Neural folds arise at the boundary between epidermis and neural plate, which is created after neural induction through the notochord (Jacobson, 1981). Neural folds, however, also arise at experimentally created boundaries between epidermis and neural plate and these new neural folds also give rise to neural crest cells (Moury and Jacobson, 1989), indicating that the notochord, although responsible for neural plate induction, is not directly involved in the induction of the neural crest. Neural crest cells originate from both the neural plate and the epidermis in both naturally occurring as well as in experimentally induced neural folds (Moury and Jacobson, 1990). Soon after formation, neural crest cells leave the neural tube, migrate throughout the embryo, and give

rise to a large number of derivatives.

Cranial neural crest

Migration of neural crest cells in the head starts in the mesencephalon before closure of the neural tube. In the prosencephalon, rhombencephalon and trunk, neural crest cells leave the neuroepithelium only after separation of the neural tube from the overlying epidermis (Raven, 1931). Many studies have been performed to create a fate-map of the amphibian cranial neural crest, using either neural crest ablation (Stone, 1922; Stone, 1926; Stone, 1929; Hörstadius and Sellman, 1946), intra- and inter-specific neural crest chimeras (Hörstadius and Sellman, 1946; Chibon, 1964; Chibon, 1966), or vital dye labelling of neural crest cells (Hörstadius and Sellman, 1946; Collazo et al., 1993). These studies demonstrated that a limited portion of the cranial neural crest, from the level of the caudal prosencephalon to the posterior rhombencephalon, contributes to the cranial and visceral skeleton. The most rostral neural crest cells do not contribute to the skeleton, although grafting experiments show that they have the potential to chondrify upon contact with endoderm (Seufert and Hall, 1990). Hörstadius and Sellman (1946) showed that cranial neural crest cells grafted into the trunk region, failed to chondrify unless pharyngeal endoderm was included in the graft, further illustrating the need for tissue interactions in neural crest cell differentiation into cartilage.

Trunk neural crest

For the axolotl embryo, it is generally agreed upon that trunk neural crest cells migrate along three major pathways: dorsally, where they form the mesenchyme of the dorsal fin, laterally (between somites and epidermis), where they give rise to pigment cells, and ventrally (between somites and neural tube), where they form the elements of the peripheral nervous system (Schroeder, 1970; MacMillan, 1976; Vogel and Model, 1977; Löfberg et al., 1980). In *Xenopus laevis* embryos, however, different opinions exist on neural crest cell migration, especially on the extend of the lateral pathway. Whereas Krotoski et al. (1988) and Collazo et al. (1993) described a minor lateral pathway, Epperlein et al. (1988) could find no evidence for cells migrating laterally. Neural crest cells migrating along the ventral route in *Xenopus* embryos, were present between the neural tube and the posterior half of the somites, thus showing a metameric pattern, whereas few cells were present within the somites (Krotoski et al., 1988). For the caudal trunk region, two additional pathways into the ventral fin have been described (Collazo et al., 1993). One group of cells migrates circumferentially within the fin, while another progresses ventrolaterally to the anus before populating the ventral fin. This latter group of cells passes through the enteric region, where they can be found temporarily.

The onset of migration in the trunk is temporally and regionally well coordinated, resulting in a wave of initiated neural crest cell migration passing along the body axis in a craniocaudal direction (Detwiler, 1937; Bancroft and Bellairs, 1976; Tosney, 1978; Löfberg

et al., 1980). Little, however, is known about the mechanisms regulating this coordinated onset of cell migration, although there is evidence indicating that factors intrinsic to the neural crest cells (Newgreen and Gibbins, 1982) as well as factors in the embryonic environment (Löfberg and Nynäs-McCoy, 1981; Erickson and Weston, 1983) may play a role. Both fibronectin and tenascin are present in the extracellular matrix lining the neural crest migratory pathways at the time when neural crest cells are actively migrating (Epperlein et al., 1988). But, whereas fibronectin is already present before the onset of neural crest migration, the appearance of tenascin is correlated with the initiation of migration, suggesting that an interaction between these two extracellular matrix components could be important in regulating the onset and pathways of neural crest cell migration. In the axolotl embryo, it has been shown that the subepidermal extracellular matrix, which forms a substrate for cell locomotion, initiates and regulates the onset of neural crest cell migration along the lateral pathway (Löfberg et al., 1985). In the white axolotl mutant, trunk pigmentation is restricted, because the epidermis is unable to support subepidermal migration of pigment cells, due to a retarded maturation of the extracellular matrix (Löfberg et al., 1989).

1.3. The neural crest in birds

The avian neural crest forms during the second day of embryonic development (E2, stage 8-16, Hamburger and Hamilton, HH; 1951) along the entire antero-posterior axis of the embryo.

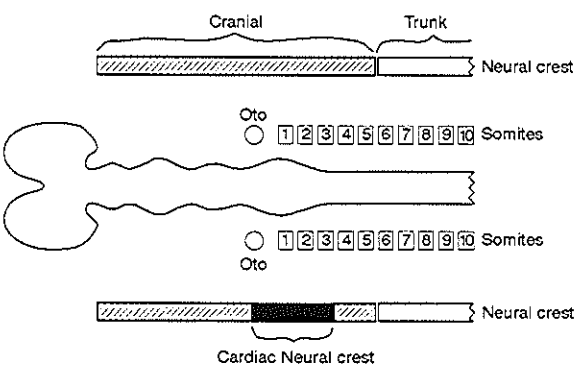

Figure 4: Diagram illustrating the various parts of the neural crest. The cranial neural crest extends from the mid-diencephalon to somite 5 and trunk neural crest begins at somite 5 and extends to the caudalmost limit of the neural tube. The cardiac neural crest, from the level of the mid-otic vesicle down to the caudal boundary of somite 3, forms a transitional region.

Depending on the axial level of origin, the neural crest can be divided into several different regions (Fig. 4), each giving rise to specific derivatives. Numerous studies have been conducted to trace neural crest cell migration and to construct a fate-map of the neural crest (see LeDouarin, 1982 for review). In these studies, a number of different techniques were used: many studies used surgically created chimeric embryos, transplanting either ^{3}H-Thymidine labelled (Weston, 1963; Johnston, 1966) or quail-derived neural crest cells (LeDouarin, 1982; Noden, 1988) to chicken embryos; other studies used the monoclonal antibody HNK-1 (or NC-1) to study early neural crest cell migration (Vincent et al., 1983; Vincent and Thiery, 1984); more recent studies used microinjection of vital dyes, like 1,1-dioctadecyl-3,3,3',3'-tetramethylincarbo-cyanine perchlorate (DiI) (Lumsden et al., 1991; Serbedzija et al., 1991) and lysinated rhodamine dextran (Bronner-Fraser and Fraser, 1988), or retroviral markers (Stocker et al., 1993), to trace neural crest cell migration. Major advantages of these latter techniques are that they do not require surgical intervention and allow for lineage analysis by injecting single neural crest cells.

Cranial neural crest

The cranial neural crest entails the neural crest overlying the diencephalon, mesencephalon and rhombencephalon, and ends at the caudal boundary of the fifth somite. The prosencephalon of avian embryos does not give rise to neural crest cells in contrast to the amphibian and mammalian forebrain. The mesencephalic neural crest cells are the first to start migrating at stage 8, after closure of the neural tube. Migration proceeds in both anterior and posterior direction. Neural crest cells of the di- and mesencephalon initially remain dorsal to the neural tube and shift rostrally over the roof of the forebrain. Subsequently, they disperse rostrally and laterally between the prosencephalic neuroepithelium and the surface ectoderm to form all of the frontonasal mass and much of the periocular mesenchyme, giving rise to cranial ganglia and contributing to the facial skeleton.

Mesencephalic neural crest cells start migration in a region rich in fibronectin, laminin, heparan sulphate proteoglycan and tenascin (Krotoski et al., 1986; Bronner-Fraser and Lallier, 1988), together forming a dense matrix surrounding and interdigitating with premigratory neural crest cells. In vivo perturbation experiments, in which antibodies against these extracellular matrix molecules or their receptors were injected in the mesencephalic region of early chicken embryos, showed that these extracellular matrix molecules were all involved in cell migration (Matthew and Patterson, 1983; Bronner-Fraser, 1985; Bronner-Fraser, 1986; Poole and Thiery, 1986; Bronner-Fraser and Lallier, 1988). Microinjection of the monoclonal antibody HNK-1 was also found to disturb cranial neural crest cell migration (Bronner-Fraser, 1987). This monoclonal antibody recognizes a carbohydrate epitope, which was first described on human natural killer cells, and was found to react with early migrating neural crest cells

and their neuronal derivatives (Vincent et al., 1983). Since then, however, it was found to be not entirely specific for these cells. It was found to be present on a large number of molecules all involved in cell-cell or cell-substrate adhesion (Kruse et al., 1984). Injection of antibodies against the cell adhesion molecules N-CAM and N-Cadherin also perturbs early cranial neural crest cell migration (Bronner-Fraser et al., 1992), further indicating that the balance between cell-cell and cell-matrix adhesion may be critical for cranial neural crest cell migration.

The rhombencephalic, or hindbrain, part of the vertebrate central nervous system is segmented, consisting of eight consecutive rhombomeres (Vaage, 1969; Keynes and Lumsden, 1990). Rhombencephalic neural crest cells migrate predominantly along a dorsolateral pathway underneath the surface ectoderm and populate the pharyngeal arches, in a way that neural crest cells of each rhombomere populate a particular pharyngeal arch which subsequently gives rise to specific derivatives. Whether or not rhombomeres 3 and 5 give rise to neural crest cells is not entirely clear (Lumsden et al., 1991; Graham et al., 1993; Sechrist et al., 1993). In this region increased cell death has been described (Lumsden et al., 1991; Jeffs et al., 1992; Jeffs and Osmond, 1992). Table 1 shows the relationship between neural crest cells of each rhombomere, the arch they populate, and the derivatives they will give rise to. Neural crest cells of the anterior rhombencephalon, ranging from the mid-hindbrain border to the mid-otic vesicle, comprising rhombomeres 1-5, populate the first two pharyngeal arches, which mainly contribute to the craniofacial skeleton, the hyoid and to cranial ganglia. The posterior rhombencephalic neural crest, overlying rhombomeres 6-8, has its anterior boundary at the level of the mid-otic vesicle. The posterior boundary of rhombomere 8 is less clear, but is thought to lie between the fifth and sixth somite. This region of the neural crest populates pharyngeal arches III, IV and VI, and gives rise to both ectomesenchymal and ganglionic derivatives. Ectomesenchymal derivatives mainly entail cells in the outflow tract of the heart and of the carotid and ultimobranchial body, and the mesenchymal component of the thymus and parathyroids. Ablation experiments showed that these ectomesenchymal derivatives originate mainly from a subregion of the posterior rhombencephalon, from the level of the mid-otic vesicle down to the caudal boundary of somite 3 (Kirby et al., 1983; Bockman and Kirby, 1984). This so-called cardiac neural crest also gives rise to ganglionic derivatives, such as cranial and cardiac ganglia (Kirby and Stewart, 1983). Migration of the cardiac neural crest has been studied extensively, both in whole-mounts (Kuratani and Kirby, 1991) and in quail-chick chimeras (Miyagawa-Tomita et al., 1991). These studies showed that cardiac neural crest cells migrate predominantly along a dorsolateral pathway, temporarily arresting to form the circumpharyngeal crest before populating the third, fourth and sixth pharyngeal arches. The migration pathways of the rhombencephalic neural crest cells caudal to the cardiac crest (adjacent to somites 4-7) have not been studied in detail.

The enteric nervous system, a ganglionic derivative of the posterior rhombencephalic neural crest, was found to derive from the vagal neural crest (LeDouarin and Teillet, 1973).

This vagal neural crest partially overlaps the posterior rhombencephalic and cardiac crest, but extends somewhat more caudally, down to the caudal boundary of somite 7. In this thesis, we present evidence that a specific subregion within the vagal neural crest, adjacent to somites 3-5, is primarily responsible for ENS development (Peters-van der Sanden et al., 1993).

Trunk neural crest

Cells from the trunk crest region, ranging from the anterior boundary of somite 6 down to the level of the tail bud, migrate along two major pathways (Serbedzija et al., 1989; Bronner-Fraser et al., 1991). The majority of the cells migrate along a ventrolateral pathway, through the somites, and give rise to the neurons and supportive cells of dorsal root and sympathetic chain ganglia, and, at the level of somites 18 to 24, to neuroendocrine cells of the adrenal medulla (LeDouarin and Teillet, 1973). Other trunk neural crest cells migrate along a dorsolateral pathway underneath the ectoderm and give rise to pigment cells.

Neural crest cells migrating along the ventrolateral pathway, emigrate from the neural tube in a continuous antero-posterior stream, but subsequently become restricted to the rostral half of each somite (Rickmann et al., 1985; Teillet et al., 1987). This metameric migration pattern results in the segmental arrangement of the dorsal root and sympathetic chain ganglia, which form aligned with the rostral half of each trunk somite (Teillet et al., 1987; Lallier and Bronner-Fraser, 1988). The metameric pattern of migration was shown to be inherent to the somites. After a 180° antero-posterior rotation of the segmental plate, which will give rise to the somites, neural crest cells migrated through the portion of the somite that was originally rostral, but was now caudal with respect to the orientation of the embryo (Stern et al., 1991b). Replacement of normal somites with only rostral halves leads to the formation of large, unsegmented ganglia (Kalcheim and Teillet, 1989; Goldstein and Kalcheim, 1991). Motor and sensory axons that grow from the neural tube also specifically go through the rostral half of the somites (Keynes and Stern, 1984). The preferential migration through the rostral half of the somites of both neural crest cells and axons could be caused either by stimulatory factors present in the rostral half or by inhibitory factors present in the caudal half of each somite. Most studies performed until now point to the presence of inhibitory factors in the caudal half, although the definite candidate has yet to be identified (Stern and Keynes, 1987; Norris et al., 1989; Stern et al., 1989; Ranscht and Bronner-Fraser, 1991). The first five somites adjacent to the posterior rhombencephalon are completely inhibitory for ganglion formation, although neural crest cell migration into these somites has been observed (Lim et al., 1987; Teillet et al., 1987). This indicates that the somitic mesenchyme can not only inhibit neural crest cell migration, but also regulates gangliogenesis.

The notochord and the perinotochordal matrix also inhibit neural crest cell migration (Newgreen et al., 1986; Pettway et al., 1990; Stern et al., 1991a). Fixation of the notochord or trypsin treatment abolishes this inhibitory effect, indicating that the responsible substance

is proteinaceous (Pettway et al., 1990). Although the notochord is known to induce ventral neural tube structures (van Straaten et al., 1985; Jessel et al., 1989; van Straaten et al., 1989), it does not prevent formation of the dorsal neural crest (Artinger and Bronner-Fraser, 1992a). Additional evidence that neural crest cell migration takes place through all available spaces unless it is restricted by inhibitory cues, comes from a study in which the neural tube was rotated around its dorso-ventral axis (Stern et al., 1991a). The neural crest cells now started migrating dorsally, showing that they do not possess intrinsic directionality in their migration, but rather exploit all those areas accessible to them and that do not inhibit their migration.

Emigration of trunk neural crest cells from the neural tube at a certain axial level occurs during a prolonged period of time. The first cells that leave the neural tube embark on the ventrolateral pathway, while the latest emigrating cells migrate dorsolaterally. Even within the ventrolateral pathway, there is a difference between the developmental potential of early and late emigrating neural crest cells (Weston and Butler, 1966). Whereas early emigrating cells migrate far ventrally to give rise to sympathetic ganglia, later emigrating cells remain in more dorsal positions and give rise to dorsal root ganglia. Transplantation of 'old' crest into a 'young' host and visa versa has shown that the last cells to leave the crest ('old' crest) have the same range of developmental capabilities in a 'young' host as early migrating cells. Within the host environment, however, some temporal alterations occur which progressively limit the distal migration of neural crest cells. Trunk neural crest cells that migrate along the dorsolateral pathway show a one-day delayed emigration from the neural tube compared to ventrolaterally migrating cells at the same axial level (Erickson et al., 1992). This delayed migration is controlled by the dermatome, removal of which results in a quicker embarkment onto the dorsolateral pathway. Once neural crest cells have entered the dorsolateral pathway, they colonize it very rapidly. These late-emigrating neural crest cells are partially restricted in their developmental potential, being no longer able to differentiate into adrenergic cells neither in vivo nor in vitro (Artinger and Bronner-Fraser, 1992b). These results show that the contribution of trunk neural crest cells to their derivatives occurs in a ventral to dorsal progression, with the precursors for pigment cells being the last to exit the neural tube.

1.4. The neural crest in mammals

Study of the neural crest in mammalian embryos has traditionally been based on descriptive morphology and extrapolation from other vertebrates, because mammalian embryos are not amenable to transplantation techniques. Although this approach may be valid for the trunk, it has serious limitations for the cranial region, because mammalian embryos have taken cranial specialization a stage further than other vertebrates. This becomes increasingly clear

as development proceeds, with the enormous expansion of the telencephalon to form the cerebral hemispheres with a true neocortex. Although there is considerable variation in the pattern of migration among mammalian embryos as a group, the basic principles of craniofacial development are very similar.

Cranial neural crest

Although some aspects of cranial neural crest cell migration are similar to those observed in avian embryos, there are variations in the exact pathways and timing of migration, and in the axial levels that contribute to the neural crest. Cranial neural crest cells in mammals emigrate from the neural folds prior to neural tube closure (Verwoerd and van Oostrom, 1979; Nichols, 1981; Nichols, 1986). Closure of the neural tube starts at the 7-somite stage at the level of somites 4 and 5, and progresses both rostrally and caudally. In the cranial region closure is complete at the 16-somite stage.

Recently, study of neural crest cell migration in the mammalian head has been made feasible through injections of WGA-Au (wheat-germ agglutinin-gold) or DiI into the amniotic cavity (Smits-van Prooije et al., 1988; Serbedzija et al., 1992). Migration starts at the 5-somite stage in the rostral hindbrain (rhombomere 1), followed by migration in the midbrain and caudal hindbrain and finally in the forebrain. In the mouse embryo, neural crest cells of the forebrain migrate ventrally in a contiguous stream through the mesenchyme between the eye and the diencephalon. Midbrain neural crest cells migrate through the mesenchyme as dispersed cells, which differs from the subectodermal stream observed in the midbrain of avian embryos (LeDouarin, 1982). In the hindbrain of mouse embryos, neural crest cells migrate in three subectodermal streams, each extending into the distal portions of the adjacent pharyngeal arches similar to the migration described in avian embryos (Lumsden et al., 1991). In the rat embryo, the forebrain does not give rise to neural crest cells, similar to avian embryos (Noden, 1975; Tan and Morris-Kay, 1985; Smits-van Prooije et al., 1988). In the midbrain of the rat embryo neural crest cells migrate as dispersed cells similar to mouse embryos. In the rat hindbrain, crest cells migrate ventrally through the mesenchyme as dispersed cells (Tan and Morris-Kay, 1986), which differs from the subectodermal streams observed in mice and avians. In the mouse embryo, all 8 rhombomeres give rise to neural crest cells (Serbedzija et al., 1992), whereas in chicken embryos there seem to be no neural crest cells migrating from rhombomeres 3 and 5 (Lumsden et al., 1991). Recent evidence, however, suggests that rhombomere 3 and to an even greater extent rhombomere 5 generate neural crest cells in avian embryos as well (Sechrist et al., 1993).

Mammalian neural crest cells were found to be able to participate in normal embryonic development after microinjection into post-implantation embryos (Jaenisch, 1985). Neural crest cells and fibroblasts injected into the amniotic cavity of early mouse embryos become dispersed into the cranial mesenchyme along normal migratory pathways, in contrast to

hepatoma cells and latex beads (Chan and Lee, 1992). This indicates that the ability to disperse along neural crest migratory pathways correlates with the ability to respond adequately to signals in regions of active cell migration and rules out passive displacement. Injection at different time-points in development showed that the migratory environment changed concomitantly with an increase in developmental age, limiting the extend of migration. Such a change in migratory environment related to developmental age was also described for the trunk neural crest of avian embryos (Bronner-Fraser and Cohen, 1980).

Trunk neural crest

Mammalian trunk neural crest cells migrate along two major pathways, one ventrolateral through the anterior part of the somites, the other dorsolateral underneath the ectoderm. Migration is very similar to avian trunk neural crest cells except for the timing. Neural crest cells appear on the dorsal surface of the neural tube 2-4 somites rostral to the most recently formed somite, whereas in avian embryos neural crest cells appear about 5 somites rostral (Erickson et al., 1989; Serbedzija et al., 1990). The ventral pathway is segregated into two phases of migration (Serbedzija et al., 1990). The early phase starts before E9 and ends at E9.5 and consists of a stream of cells within the rostral sclerotome, which extends ventrally to the region of the sympathetic ganglia. The later phase starts after E9.5 through E10.5 and consists of a thin strand of cells along the lateral surface of the neural tube, which later protrudes into the rostral sclerotome to form dorsal root ganglia and Schwann cells. At all stages during migration, crest cells are found on the dorsolateral pathway, in contrast to avians.

1.5. Patterning of the rhombencephalic neural crest

Neural crest cells emigrating from the rhombencephalon, populate the pharyngeal arches and contribute to a large variety of derivatives, including cranial ganglia, the facial skeleton, the outflow septum of the heart, the mesenchymal component of thymus and parathyroids, and the ENS. The rhombencephalon has received much attention in recent years because the properties of its neural crest cells suggest that they may have a leading role in the patterning of structures at this axial level (Noden, 1988; Richman and Tickle, 1989; Noden, 1993). When premigratory neural crest from the first arch is used to replace the premigratory crest of the second arch of a host embryo, the grafted crest migrates in a way appropriate for its new position and enters the second arch. Once there, however, it makes a set of structures appropriate for its original position, i.e. first arch jaw cartilage in the second arch (Noden, 1983; Noden, 1988). Furthermore, it is able to influence the surrounding non-neural crest derived tissues, i.e. surface ectoderm and paraxial mesoderm to form first arch structures

(beaks and jaw muscles, respectively). This suggests that neural crest cells of the rhombencephalon acquire regional identity while still being part of the neuroepithelium, that they carry this identity with them as they move into the pharyngeal arches, and are able to transmit it to their surroundings (Noden, 1988).

The discovery of vertebrate *Antennapedia* class homeobox-containing (*Hox*) genes has led to a better understanding of some of the molecular processes in vertebrate hindbrain development (Akam, 1989; McGinnis and Krumlauf, 1992). *Hox* genes, containing a 180 bp homeodomain, encode a group of sequence specific proteins, which are capable of binding to the DNA. These proteins can act as transcription factors and have been implicated in many

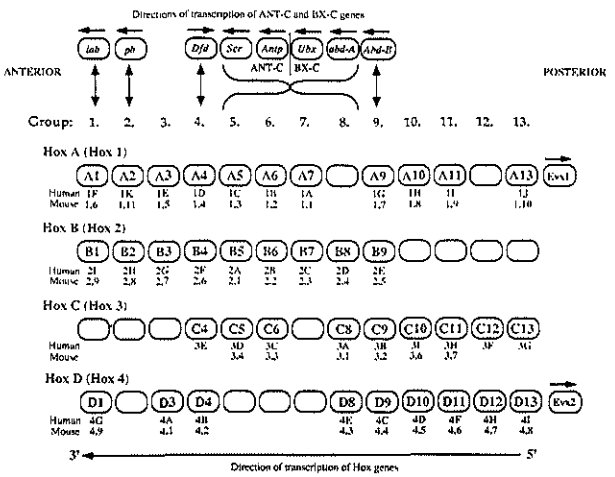

***Figure 5**: Four mammalian Hox complexes and the new and old names of the genes. The new names are a single letter (A, B, C, or D) followed by a number from 1 to 13. Genes expressed most anteriorly have the lowest numbers. The new numbers (in ovals) are shown in the order they are found along the chromosome.The most commonly used synonyms are shown below each oval. Each column of genes indicates corresponding genes in the four Hox complexes based on homeodomain sequences alone. Empty ovals indicate that no gene has been detected in mice or humans. To date, all Hox genes including (excluding Evx genes) appear to be transcribed in the direction shown at the bottom of the figure. Alignment with Drosophila genes is shown at the top. A strong case can be made from sequence similarity and expression pattern for the relatedness of labial (lab), proboscipedia (pb), Deformed (Dfd), and Abdominal-B (Abd-B) with groups 1, 2, 4, and 9, respectively. There are four groups in the region corresponding to sex combs reduced (scr), Antennapedia (Antp), Ultrabithorax (Ubx), and Abdominal-A (Abd-A), but more exact relations have not been determined (indicated by brackets). Short arrows indicate the directions of transcription of genes in ANT-C and BX-C. (From M.P. Scott, 1992, Cell 71:551-553)*

aspects of development. *Hox* genes were found to be arranged in clusters along the chromosome. During the evolution of chordates, the number of *Hox* gene clusters has increased, probably through chromosomal duplication events. The acorn worm (a hemichordate) has only one *Hox* cluster, two were found in amphioxus (a cephalochordate), whereas in the lamprey (a primitive vertebrate) three *Hox* clusters were identified (Pendleton et al., 1993). Vertebrates possess four clusters of *Hox* genes, which are known in mammals as the *Hox-A, Hox-B, Hox-C,* and *Hox-D* (formerly called *Hox-1* through *4*) gene clusters; Fig. 5). It is possible to identify subfamilies of up to four genes each belonging to a different cluster (known as paralogous groups), which presumably arose from a single ancestor gene. There is, however, not a one to one relationship between genes in the various clusters, because of tandem duplications of genes within a cluster (Krumlauf, 1992). Genes within one cluster display a direct linear relationship between their order along the chromosome and the antero-posterior axial level at which they are expressed. Genes that lie most 3' within a *Hox* gene cluster, have the most anterior expression restriction with cutoffs corresponding to rhombomere boundaries (Wilkinson et al., 1989). This colinearity was first described in *Drosophila* (Lewis, 1978), but was also found in vertebrates (Duboule and Dollé, 1989; Graham et al., 1989). Expression of a specific combination of *Hox* genes within each rhombomere results in a segment restricted *Hox* code (Hunt et al., 1991a). This code is first established in the neuroepithelium when still containing the neural crest and is maintained during neural crest cell migration. This results in a *Hox* code in the cranial ganglia and the pharyngeal mesenchyme, reflecting their rhombomere of origin (Lumsden et al., 1991). Upon contact with the ectoderm, the pharyngeal mesenchyme transduces its *Hox* label, consistent with the evidence that the neural crest is able to influence the development of other tissues in the head (Hunt et al., 1991b).

In order to really prove that the expression of a particular combination of *Hox* genes could be responsible for controlling the identity of a segment, the cranial *Hox* code has been experimentally altered. The technique of homologous recombination in embryonic stem cells was used to produce mice lacking functional copies of genes of the *Hox-A* cluster. Mice lacking the *Hox-A1* or the *Hox-A3* gene died shortly after birth and showed defects in the pharyngeal region (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992). Defects in *Hox-A3* mutants occurred mainly in mesenchymal derivatives of the rhombencephalon (Chisaka and Capecchi, 1991), whereas *Hox-A1* mutants mainly displayed abnormal development of neural structures (Lufkin et al., 1991; Chisaka et al., 1992). A significant feature of the phenotypes of both mutants was, that while there were profound effects on the development of structures at a particular axial level, broadly correlating with the anterior domain of expression of the genes, other structures deriving from the same axial level, which in some cases expressed the gene in normal development, were unaffected. Overexpression of the *Hox-A4* gene also resulted in a defective development of

rhombencephalic structures (Wolgemuth et al., 1989). These mice suffered from megacolon caused by a non-functional ENS (Gershon and Tennyson, 1991).

Defective development of rhombencephalic structures was also observed after fetal exposure to retinoic acid (RA), both in humans (Lammer et al., 1985) and in a number of animal species (Shenefelt, 1972; Fantel et al., 1977; Kamm, 1982; Webster et al., 1986). The phenotype somewhat resembled the *Hox-A3* knock-out mice with a disturbed development of the mesenchymal derivatives of the rhombencephalon, i.e. absence or hypoplasia of the thymus and parathyroids and impaired outflow septation of the heart (not found in the *Hox-A3* mutants). Recently, it was shown that RA is capable of altering the hindbrain *Hox* code, resulting in the homeotic transformation of rhombomeres 2/3 to a 4/5 identity (Marshall et al., 1992). After entrance into the cell and reaching the nucleus, RA can form a complex with specific RA receptors. This RA-receptor complex acts as a transcription factor and is able to regulate gene expression through binding to RA-responsive elements present in the promoter region of certain genes. Two cellular RA binding proteins (CRABP-I and CRABP-II), present in the cytoplasm, are thought to modify the effect of RA. They could either function as a shuttle to transport RA to the nucleus, or they could regulate the concentration of free cytoplasmic RA either by steepening a RA gradient or by functioning as a sink, protecting the nucleus from excess RA. The latter possibility is favoured by a study in F9 teratocarcinoma cells in which CRABP-I was shown to influence the metabolism of intracellular RA (Boylan and Gudas, 1992). Additional evidence for a protective role of CRABP-I against excess RA comes from the fact that CRABP-I has been found to be specifically expressed in tissues that seem to be sensitive to RA exposure during development (Vaessen et al., 1990; Maden et al., 1991; Ruberte et al., 1991).

1.6. In vitro studies of the neural crest

The analysis of neural crest cell migration, their state of determination and the environmental factors involved in their differentiation, has been greatly facilitated by in vitro approaches. Although these studies have been conducted in a number of different species, such as amphibians (Akira and Ide, 1987; Wilson and Milos, 1987), reptiles (Hou and Takeuchi, 1992), and mammals (Ito and Takeuchi, 1984; Ito et al., 1988; Ito et al., 1993), our main focus will be on birds.

To study factors influencing migration, neural crest cells of various axial levels were grown in vitro. The neural tube containing the premigratory neural crest was explanted to allow neural crest cells to emigrate from the dorsal aspect of the neural tube onto permissive two-dimensional substrates. Neural crest cells were found to migrate avidly on planar substrates comprised of purified extracellular matrix components such as fibronectin (Rovasio

et al., 1983), laminin (Newgreen, 1984), collagen (Cohen and Konigsberg, 1975) and tenascin (Halfter et al., 1989). Fibronectin, found to be the most adhesive molecule, is present in high concentration in the neural crest cell migration pathways (Newgreen and Thiery, 1980; Duband et al., 1986). Hyaluronate and chondroitin sulphate were found to be poor two-dimensional migration substrates (Erickson and Turley, 1983), although addition of hyaluronate to three-dimensional matrices of collagen or fibronectin increased neural crest cell migration by opening up spaces between the collagen fibrils in the gel (Tucker and Erickson, 1984). Hyaluronic acid also influences adhesivity among neuroepithelial cells and could therefore be important in the initial separation of neural crest cells from the neural tube (Luckenbill-Edds and Carrington, 1988).

An important and still largely unanswered question concerns the developmental potential of individual neural crest cells. Two major scenarios have been proposed to account for the diversity of derivatives arising from the neural crest. First, the neural crest may be composed of a homogeneous population of totipotent cells with identical developmental potential, the fate of which thought to be completely determined by the embryonic environment. A second possibility is that the neural crest may be comprised of a heterogeneous mixture of predetermined cells. These committed cells would differentiate according to an inherent program upon reaching their proper location; those in inappropriate sites either fail to differentiate or die. Evidence in support of both schemes has been obtained. In clonal analysis studies, some neural crest cells have been shown to contribute to multiple phenotypes in vitro (Bronner-Fraser et al., 1980; Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Sieber-Blum, 1989; Baroffio et al., 1991; Ito and Sieber-Blum, 1991; Ito and Sieber-Blum, 1993). Several monoclonal antibodies have been identified, however, that specifically recognize subpopulations of early migrating neural crest cells (Ciment and Weston, 1982; Girdlestone and Weston, 1985; Barbu et al., 1986; Barald, 1988), suggesting heterogeneity in early neural crest cell populations.

Clonal analysis of the posterior rhombencephalic neural crest showed that it consists of a heterogeneous mixture of both pluripotent and developmentally restricted progenitors (Ito and Sieber-Blum, 1991). Pluripotent progenitors gave rise to sensory and serotonergic neurons, chondrocytes, and connective tissue, smooth muscle and pigment cells. Upon entry into the posterior pharyngeal arches, cells lost the potential to differentiate into pigment cells and sensory neurons (Ito and Sieber-Blum, 1993), confirming earlier observations by Ciment et al. (Ciment and Weston, 1983; Ciment and Weston, 1985). The developmental potential to form serotonergic neurons, which may constitute precursors for enteric neurons, also decreased considerably upon entry into the pharyngeal arches (Ito and Sieber-Blum, 1993). These results show that although the posterior rhombencephalic neural crest contains pluripotent precursors which can give rise to serotonergic neurons, most precursors loose this capacity upon entry into the pharyngeal arches, resulting in a high percentage of clones which

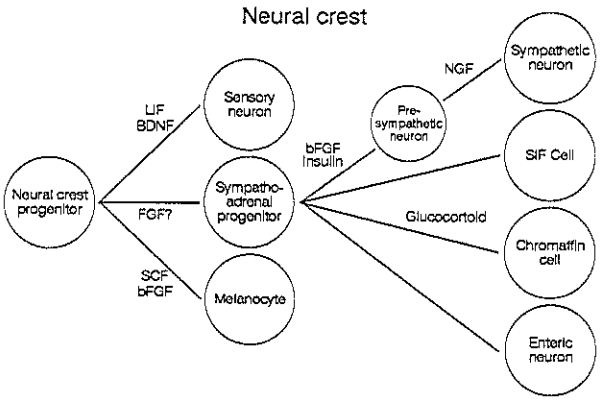

Figure 6: *Actions of growth factors on the differentiation of neural crest cells in vertebrates. A single growth factor can regulate the differentiation of many distinct cell types at different stages of embryonic development. Conversely, members of different classes of growth factors can act on the same group of progenitor cells to induce similar developmental programs. (b)FGF, (basic) fibroblast growth factor; SCF, stem cell factor; LIF, leukemia inhibitory factor; BDNF, brain derived neurotrophic factor; NGF, nerve growth factor; SIF cell, small intensely fluorescent cell.*

only consist of ectomesenchymal cell types.

The embryonic microenvironment may play an important role in the emergence of phenotypic diversity. Certain factors could act to promote the survival of selected subpopulations of fully determined progenitors, while others may direct partly committed precursors toward a specific developmental fate (Howard and Bronner-Fraser, 1985; Howard, 1986; Ziller et al., 1987; Barald, 1989). Differentiation can be influenced both through direct contact with tissues or extracellular matrix and through diffusible factors (Fig. 6).

1.7. Clinical disorders related to the neural crest

Of all the described human congenital malformations (defined as structural defects present at birth), about one third entails structures related to the neural crest (Table 1). In 1974 Bolande (1974) introduced the term neurocristopathies for certain tumors, such as pheochromocytoma, neuroblastoma, and neurofibromatosis type 1 and 2, occurring either isolated or in combination. In this way, he attempted to delineate a common pathogenetic denominator for a heterogeneous group of disorders, which would be aberrant neural crest development. Since then, the term neurocristopathy has been used for any malformation concerning structures that receive some contribution from the neural crest.

Table 2: Pharyngeal arch related syndromes and their characteristic defects

Syndromes	DiGeorge	VCF[1]	Goldberg-Sphrintzen	VACTERL	Goldenhar	CHARGE	FHD[2]
Defects							
Related to arches I/II	Micrognathia (Fishmouth)	Micrognathia	Micrognathia		Micrognathia Uni- or bilateral facial hypoplasia Eye defects	Micrognathia	
Related to arches III/IV	Ear defects Vascular defects Heart defects Thymus aplasia Thyroid defects Parathyroid defects	Ear defects Vascular defects Heart defects	Ear defects Vascular defects Heart defects	Vascular defects Heart defects	Microtia Heart defects	Ear defects Heart defects	Heart defects
Others	Vertebral defects Cerebral defects[3]	Cerebral defects[3] Deafness[3] Prominent nose Broad nasal root	Cerebral defects[3] Deafness[3] ENS defects[4] Prominent nose Broad nasal root	Vertebral defects Anal atresia Tracheal fistula Esophageal fistula Renal defects Limb defects	Vertebral defects	Cerebral defects Deafness Choanal atresia Coloboma Genital defects	

[1]) Velocardiofacial or Sphrintzen syndrome
[2]) Familial heart disease
[3]) not always found
[4]) Disorders of the enteric nervous system

As has become apparent in this chapter, the neural crest contributes to a vast amount of structures throughout the body. Most of these structures, however, are not entirely neural crest derived, but involve interactions with other germ-layers. Ear development, for example, involves interactions between ectoderm (first pharyngeal cleft and otic placode), mesoderm (mostly neural crest cells from pharyngeal arches 1-4) and endoderm (first pharyngeal pouch), each giving rise to a specific structure of ear (Larsen, 1993). Furthermore, complex malformations often occur in syndromes, with a characteristic set of malformations occurring in the same patient. Waardenburg type I syndrome, for example, is characterized by congenital deafness, pigment abnormalities (white forelock) and various other anatomical changes (Omenn and McKusick, 1979; Badner and Chakravarti, 1990). It is caused by a mutation in the *PAX-3* gene, which, in mice, was found to be expressed in the dorsal part of the neural tube, including the neural crest (Baldwin et al., 1992; Gruss and Walther, 1992; Tassabehji et al., 1992). Although part of the malformations in this syndrome could be caused by a genetic defect in the neural crest, not all of the malformations can be explained by defective neural crest development.

Many syndromes which are considered to be neurocristopathies, like Waardenburg type I syndrome, involve structures in the head and neck region, which are related to the pharyngeal arches. Syndromes related to the third and fourth pharyngeal arches may entail malformations of the outflow tract of the heart, the thymus, the parathyroids, and the ENS, often combined with craniofacial dysmorphologies. Well-known examples are the DiGeorge, Goldenhar, Velocardiofacial and Goldberg-Sphrintzen syndromes as well as the CHARGE association (Table 2). Careful examination of Table 2 shows that there is considerable overlap in the malformations found in these syndromes. This shows the difficulty of syndrome delineation and stresses the importance of careful examination of patients.

1.8. Conclusions

Comparing neural crest development in various vertebrate species shows that, although there are species specific differences in both the timing and pathways of neural crest cell migration and differentiation, the basic principles are very similar. We chose the chicken embryo as a model system in the experimental work, because, like amphibians, it is easily amenable to experimental manipulation. Amphibians, however, differ from birds and mammals in the extend of cephalization, which can be illustrated by the evolution of the skull (Augier, 1931; Couly et al., 1993). Primitive vertebrates, such as the agnatha, have a small archiskull in which no vertebrae participate, whereas higher vertebrates, such as birds and mammals, have a neoskull in which 5 somites participate in the occipital bone complex. Amphibians have an intermediate skull type, a paleoskull, in which 3 somites are incorporated. This difference in

skull type has consequences for the neural crest of somites 4 and 5, which belongs to the cranial crest in birds and mammals, but should be considered trunk crest in amphibians. The chicken embryo is also well suited to study the role of various growth factors on neural crest cell differentiation in vitro.

Although most studies support the similarity of neural crest development in birds and mammals, one should remain careful in the extrapolation of data. This can be illustrated by comparing certain pharyngeal arch derivatives. In birds, the cartilages of the first pharyngeal arch give rise to the jaw joint. Among the immediate ancestors of mammals, however, a second, novel jaw articulation developed. As a result, the cartilage that formed the jaw joint in non-mammals, shifted to the middle ear and joined with the preexisting stapes (derived from the cartilage of the second pharyngeal arch) to form the unique three-ossicle auditory mechanism of mammals (Larsen, 1993).

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Chapter 2

Introduction to the enteric nervous system

2.1. Evolutionary aspects of intestinal motility

The development of specialized food-transport systems preceded the origin of vertebrates and the vertebrate neural crest. Early prevertebrates were small, marine, filter-feeding organisms, which had no specialized food-transport mechanisms and depended solely on diffusion for their nutrition. But with the development of a muscular pharynx in protochordates, the first primitive food-transport system became available. The functioning of such a system can be illustrated by the pharyngeal apparatus in the nematode *Caenorhabditis elegans*. The pharynx of *C. elegans*, which feeds on bacteria, consists of three functional parts (Fig. 7) (Avery and Horvitz, 1989). The first part is formed by the corpus, which consists of two types of large muscles, i.e. the procorpus (M3) and the metacorpus (M4). The second part consists of one

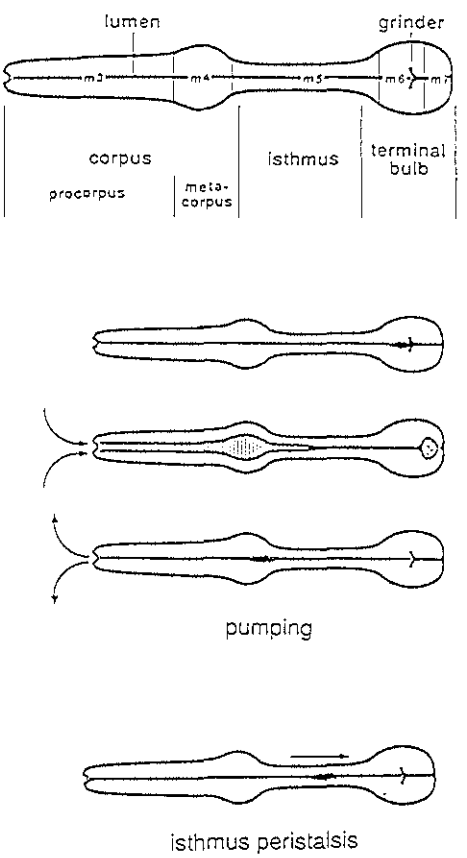

***Figure** 7: Anatomy and function of the pharynx of C. Elegans. A) The pharynx is divided into three functional parts, the corpus, the isthmus, and the terminal bulb. The corpus is subdivided into the procorpus and metacorpus. There are five types of large muscles in the pharynx, arranged from anterior to posterior: m3 in the procorpus, m4 in the metacorpus, m5 in the isthmus, and m6 and m7 in the terminal bulb. B) Pumping: a pump consists of a nearly simultaneous contraction of the corpus, anterior isthmus, and terminal bulb, followed by relaxation. Corpus and isthmus muscles are radially orientated, so the lumen opens when they contract, sucking in liquid and suspended bacteria. Terminal bulb muscle contraction inverts the grinder, breaking bacteria that are in front of the grinder, and passing debris back to the intestine. Relaxation returns the grinder to its relaxed position and allows the lumen of the corpus to close, expelling liquid. Bacteria are trapped in a filter in the back of the corpus. C) Isthmus peristalsis: the feeding cycle is closed by a peristaltic contraction of the posterior isthmus muscles, which carries bacteria from the back of the corpus to the grinder. Anterior is to the left. (From L. Avery and H.R. Horvitz, 1989, Neuron 3:473-485)*

large muscle called the isthmus (M5). The third part consists of two muscles (M6 and M7) and is called the terminal bulb. Feeding is accomplished by two separately controlled muscle motions: pumping and isthmus peristalsis. During a pump all muscles of the pharynx contract nearly simultaneously followed by relaxation. Because of the radial orientation of M3-5, this contraction leads to the opening of the lumen of the corpus and anterior isthmus, whereas the posterior isthmus remains closed (Albertson and Thomson, 1976). This results in the sucking in of liquid and suspended bacteria, which accumulate in the back of the corpus (Seymour et al., 1983). During the same pump the terminal bulb contracts, resulting in the crushing of bacteria that are in front of the grinder, a specialized structure in the terminal bulb. The debris is subsequently passed back to the corpus (Doncaster, 1962). Bacteria were transported from the corpus to the grinder during a previous feeding cycle which ended with a peristaltic contraction of the posterior isthmus muscles (isthmus peristalsis). All these coordinated pharyngeal muscle contractions are regulated by the pharyngeal nervous system consisting of 14 types of neurons, adding up to a total of 20 neurons (Albertson and Thomson, 1976). It has been found, however, that the muscles involved in pumping can function autonomously without any pharyngeal neurons being present, whereas for isthmus peristalsis the presence of only one specific neuron is needed (Avery and Horvitz, 1989). Absence of this neuron leads to an accumulation of bacteria in the corpus, resulting in stuffed larvae (a phenotype somewhat resembling megacolon in vertebrates).

These data indicate that the main role of the pharyngeal nervous system in *C. elegans* is merely to regulate the frequency of pumps and the precise timing of muscle contractions in response to environmental and physiological cues, without being necessary for pharyngeal function itself. Therefore this pharyngeal nervous system should be considered more of a CNS-related extrinsic nervous system than a neural crest-related intrinsic nervous system.

A far more elaborate food-transport system, consisting of a fore-, mid- and hindgut, is found in insects. The fore- and hindgut are derived from ectodermal invaginations that occur soon after gastrulation, whereas the midgut may receive contributions from different embryonic germ layers including ectoderm, mesoderm and endoderm (Campos-Ortega and Hartenstein, 1985). The development of the ENS in insects was studied most thoroughly in the moth (*Manduca sexta*). The ENS consists of two small ganglia (the frontal and the hypocerebral ganglion) lying dorsally on the foregut and an enteric plexus at the foregut-midgut boundary, which are connected via the recurrent nerve (Copenhaver and Taghert, 1989). From these ganglia a nerve network arises that runs superficially along the rest of the alimentary tract (Fig. 8). The two ganglia of the foregut and the recurrent nerve are derived from three neurogenic zones in the foregut epithelium which differentiate shortly after foregut formation and give rise to chains of cells that emerge onto the foregut surface (Copenhaver and Taghert, 1991). As these cells emerge from the epithelium, they briefly become mitotically active, dividing once or twice, and then gradually coalesce into the ganglia and

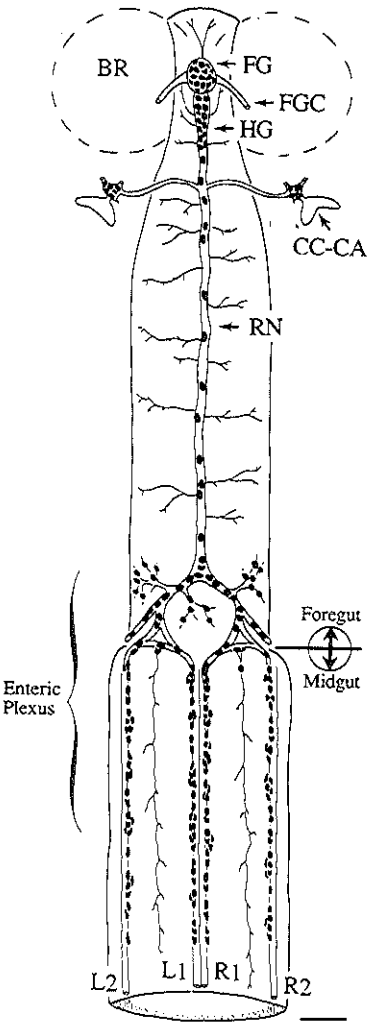

Figure 8: Cellular domains within the ENS of Manduca sexta. Two distinct populations of enteric neurons can be distinguished by their position and organization. One group of about 70 neurons forms a pair of small ganglia on the anterior surface of the foregut: the frontal ganglion (FG), which is connected to the brain lobes (BR) via the frontal ganglion connections (FGC), and the hypocerebral ganglion (HG), which is continuous with the recurrent nerve (RN) that lies mid-dorsally on the foregut surface. Paired nerves also connect the recurrent nerve to the neurohemal organs of the brain, the corpora cardiaca-corpora allata (CC-CA). A scond group of about 300 neurons (the EP cells) occupies the enteric plexus, a branching set of nerves that extend along eight longitudinal muscle bands on the midgut (only the dorsal muscle bands are shown: L1-L2 and R1-R2). (From P.F. Copenhaver, 1993, Development 117:59-74)

nerves of the anterior ENS. Enteric glial cells are also generated from these neurogenic zones as a distinct population of precursor cells (Copenhaver, 1993). These cells, which are among the last to emerge from zones 2 and 3, remain mitotically active for a prolonged period of time. At the end of this phase of neurogenesis, an ectodermal placode invaginates from the foregut epithelium, at the site of the third neurogenic zone (Copenhaver and Taghert, 1990; Copenhaver and Taghert, 1991). This ectodermal placode forms a packet of enteric plexus cells in the form of a triangular cluster at the foregut-midgut boundary with its anterior tip connected to the recurrent nerve. Subsequently, this cluster of cells is transformed into a

mature enteric plexus by a sequence of migratory events. During the first slow phase of migration, which is circumferentially directed, the triangular cluster spreads bilaterally as a cell sheath around both sides of the foregut. In the subsequent fast phase of migration the packet becomes disrupted by the abrupt dispersal of small subgroups of presumptive neurons that stream out along an array of pathways on both the fore- and midgut to innervate the visceral musculature (Copenhaver and Taghert, 1989). The dispersal of glial cells proceeds along these same pathways (Copenhaver, 1993). The pattern of cell migration observed during the formation of the ENS in the moth shares several important features with the migratory behavior of cells derived from the vertebrate neural crest: migration occurs in multiple phases (Tosney, 1978; Thiery et al., 1985; Newgreen and Erickson, 1986) and cells do not necessarily follow particular pathways, but choose their pathway by reference to local cues encountered in the course of their dispersal (Tosney, 1978; LeDouarin et al., 1984; Gershon, 1987; Copenhaver and Taghert, 1989). This shows that formation of the ENS in insects, which lack the vertebrate neural crest, involves a developmental strategy which is clearly distinct from neurogenesis in the insect CNS and closely resembles the generation of enteric neurons in vertebrates.

2.2. Structure and ultrastructure of the enteric nervous system

The vertebrate alimentary tract contains a number of specialized regions each involved in a particular function (Fig. 9). Although the general build up of the various regions of the alimentary tract are the same, there are specific differences between the various vertebrate species related to dietary variation (herbivore, carnivore or omnivore). The morphology of the gut wall varies between the different regions of the gut within one species and within one region of different species, but in general consists of the luminal epithelium, mucosa, submucosa, circular smooth muscle layer, longitudinal smooth muscle layer and serosal epithelium (Fig. 10) (Furness and Costa, 1980).

The ENS of vertebrates is made up of an extrinsic and an intrinsic component. The extrinsic component consists of a parasympathetic and a sympathetic division. The parasympathetic innervation derives from the vagal nerve and is capable of enhancing peristaltic activity. The sympathetic innervation derives from the splanchnic nerves and terminates in the large abdominal autonomic plexuses, such as the celiac plexus innervating the stomach and small intestine. This division of the extrinsic nervous system has a general suppressive action on peristaltic activity.

The intrinsic component of the ENS consists of nerve plexuses and their interconnecting fibers, embedded in the wall of the gut. The two principal plexuses are the myenteric (Auerbach) and submucous (Meissner) plexuses. The myenteric plexus is confined

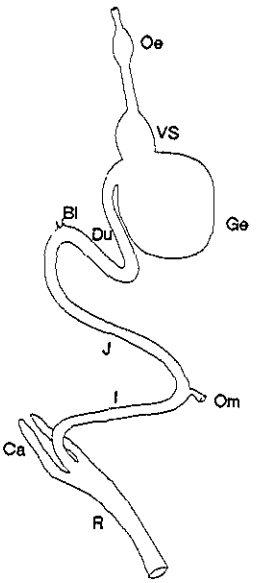

Figure 9: Schematic drawing of the chicken digestive tract, indicating its various parts. Oe= esophagus; VS= proventriculus; Ge= gizzard; Du= duodenum; Bi= bile duct; J= jejunum; Om= umbilicus; I= ileum; Ca= ceca; R= rectum.

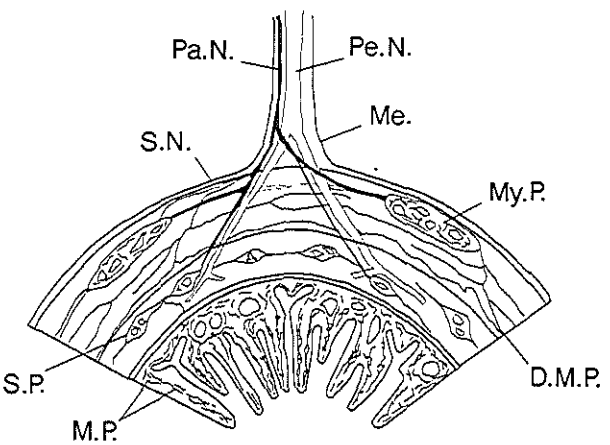

Figure 10: Diagram of the arrangement of the enteric nervous system in a transverse section of the bowel. Me.=mesentery; M.P.=mucosal plexus; Pa.N.=paravascular nerve; Pe.N.=perivascular nerve; S.N.=subserous nerve; S.P.=submucous plexus. (From Furness and Costa, 1980)

to the space between the circular and longitudinal muscle layers and, because of this position, undergoes enormous variations in size and shape during intestinal contraction. Its ganglia generally contain a large number of nerve cell bodies, which are contained in a meshwork that is quite characteristic and can be readily identified in any one area from a particular species on account of the thickness of the main nerve bundles and their branching pattern. The submucous plexus lies embedded in the connective tissue between the circular smooth muscle layer and the mucosa. Compared to the myenteric plexus, ganglia are smaller, containing fewer neurons, whereas the meshes are larger and more irregular. Sometimes two plexuses can be recognized within the submucosa called the plexus of Henle and the plexus of Meissner, which can be physically separated. The plexus of Henle appears to be a motor plexus as it contains neurons which closely resemble those found in the myenteric plexus. The plexus of Meissner is composed of small neurons resembling the neurons of the cerebro-spinal ganglia and which therefore appear to be sensory. In both the myenteric and the submucous plexuses, there are considerable variations in the size and shape of the ganglia and meshes, not only between different species, but also between different regions of the gut within one species, and between the mesenteric and the anti-mesenteric side within one region (Weyns, 1988).

The ultrastructure of the ENS differs considerably from that of other autonomic ganglia and in many respects resembles more closely the CNS. The ENS contains a large number of neurons and a remarkable diversity of neuronal cell types. The perikarya are irregularly shaped and characterized by large eccentrically placed nuclei with fine granular nucleoplasm, prominent nucleoli and sparse condensations of chromatin. The ENS also contains numerous glial cells which closely resemble CNS astrocytes, not Schwann cells (Gabella, 1971; Cook and Burnstock, 1976b), containing more glial fibrillary acidic protein (Björklund et al., 1984; Rothman et al., 1986) and extending more processes (Erde et al., 1985). It has been suggested that the enteric glial cells may be important in conferring structural stability to the ganglia, holding them together and at the same time allowing structural rearrangement of the ganglia during muscle contraction. In contrast to axons of peripheral nerves, enteric axons are not individually enveloped by a glial process, but bundles of enteric axons are invested by glial processes so that neurites abut on one another. Within the enteric ganglia, perikarya and glial cells are tightly packed with a virtual absence of an intraganglionic extracellular space, whereas other autonomic ganglia are loosely organized containing collagen, blood vessels, fibroblasts, macrophages and mast cells in addition to neurons and supporting cells. Finally, enteric ganglia are isolated from the surrounding tissue by a basal lamina which develops after complete formation of the ganglia. This basal lamina may function as a blood-plexus boundary, analogous to the blood-brain barrier. As already stated, the ENS harbors a large variety of neurons, which have been classified

Table 3: Putative and established neurotransmitters in the ENS

Class	Subclass	Reference
Acetylcholine		
Noradrenaline		
Biogene amines	serotonin	(Costa et al., 1982; Furness and Costa, 1982)
	norepinephrine	(Burnstock and Costa, 1975)
Amino acids	GABA (gamma-aminobutyric acid)	(Jessen et al., 1986)
Purines	ATP	(Burnstock, 1972)
Neuropeptides	Substance P	(Costa et al., 1981)
	VIP (vasoactive intestinal polypeptide)	(Furness et al., 1981; Costa and Furness, 1983)
	peptide histidine-isoleucine	(Bishop et al., 1984)
	somatostatin	(Costa et al., 1980; Furness et al., 1980)
	calcitonin gene-related peptide	(Rodrigo et al., 1985)
	neuropeptide Y	(Furness et al., 1983a; Daniel et al., 1985)
	pancreatic polypeptide	(Furness et al., 1983a)
	enkephalins	(Furness et al., 1983b; Costa et al., 1985)
	beta-endorphin	(Wolter, 1985a)
	dynorphin	(Dalsgaard et al., 1983; Costa et al., 1985)
	adreno cortico trophin	(Wolter, 1985b)
	alpha-melano trophin	(Wolter, 1985b)
	galanin	(Ekblad et al., 1985; Melander et al., 1985)
	cholecystokin	(Hutchison et al., 1981)
	gastrin-releasing peptide bombesin	(Hutchison et al., 1981; Costa et al., 1984)
	arginine vasopressin	(Hanley et al., 1984)

morphologically (Dogiel, 1895; Gunn, 1959; Gunn, 1968; Baumgarten et al., 1970; Feher and Vajda, 1972; Cook and Burnstock, 1976a). These different neurons contain an abundance of putative and established neurotransmitters, not seen in any other region of the autonomic nervous system and most of which are also active in the brain (Table 3) (Furness and Costa, 1980; Gershon, 1981; Gershon and Erde, 1981). Besides the two classical neurotransmitters, acetylcholine and noradrenaline, there is a large and complex peptidergic system distributed throughout the lengths of the gut. More recent evidence for the coexistence of different substances in various combinations within the same neuron, have made the picture of enteric innervation even more complex (Makhlouf, 1985). The presence of a large variety of neuronal cell types with a characteristic distribution pattern within the gut, and with a well-defined set of actions, suggests that the ENS could function as a 'mini-brain', under the general influence of the CNS, but able to function by itself.

2.3. Development of the enteric nervous system in amphibians

Development of the ENS in amphibians has been studied most extensively in *Xenopus* by constructing interspecific chimeras between *Xenopus laevis* and *Xenopus borealis* embryos (Sadaghiani and Thiébaud, 1987; Epperlein et al., 1990). In this way, it has been established that the enteric neurons originate from the neural crest adjacent to the posterior rhombencephalon and the anterior spinal cord (somites 1 and 2). These neural crest cells migrate along a ventromedial pathway between the somites and the neural tube on their way to the primitive gut (Epperlein et al., 1990). Sadaghiani and Thiébaud (1987) showed that these cells are first found scattered in a region between the last branchial arch and the posterior part of the pronephros, and subsequently migrate along dorsal root fibers and through the body of the vagus ganglion complex. They continue migration from the lower part of this ganglion complex by means of the recurrent-intestinal nerve, which runs over the glottis musculature, and penetrates the wall of the esophagus and larynx. Then they translocate craniocaudally through the splanchnopleural layer of the intestinal tract and its accessory glands and differentiate into mature enteric neurons.

2.4. Development of the enteric nervous system in birds

The avian gut is formed by lateral infolding of the endoderm and the splanchnic mesodermal epithelium. This results in the formation of a tube, consisting of a lumen surrounded by endodermal and splanchnic epithelia. The mesenchymal cells of the gut are formed through de-epithelialization of cells from the splanchnic epithelium, thus forming a loose mesenchyme

between the two epithelia which later gives rise to the muscles and connective tissue of the gut.

The origin of the enteric neurons has long been an issue of debate (see (Andrew, 1971) for review), but it is now generally agreed upon that the enteric neurons are mainly derived from the vagal neural crest (Yntema and Hammond, 1954; LeDouarin and Teillet, 1973; Allan and Newgreen, 1980). In their fate-map study, LeDouarin and Teillet (1973) also found evidence for a contribution of the lumbosacral neural crest, caudal to somite 28, to the ENS in the postumbilical gut. This sacral contribution to the postumbilical ENS has since then been confirmed in a number of studies (Pomeranz and Gershon, 1990; Pomeranz et al., 1991a; Serbedzija et al., 1991), but its precise fate has not yet been established. It has been shown that in the absence of vagal neural crest cells, sacral neural crest cells are not capable of giving rise to enteric ganglia (Yntema and Hammond, 1954) (Peters-van der Sanden et al., unpublished). The sacral neural crest does give rise to the ganglion of Remak (Yntema and Hammond, 1953; Yntema and Hammond, 1955; Teillet, 1978), a ganglionated nerve which is only present in birds and which belongs to the autonomous nervous system. It runs parallel to the gut in the mesentery and extends from the duodenal-jejunal junction, where it is connected with the celiac plexus, to the cloaca, where it joins the pelvic plexus (Nolf, 1934).

Although the previous paragraph indicates that the vagal neural crest is the principal source for enteric neurons in vivo, there is some debate whether other axial levels of the neural crest may also be capable of giving rise to enteric ganglia in an experimental system. In a number of studies, aneural hindgut, not containing neural crest cells, was cocultured with neural crest segments derived from various axial levels, on the chorioallantoic membrane of a host embryo (LeDouarin and Teillet, 1974; Smith et al., 1977; Teillet et al., 1978; Newgreen et al., 1980; Smith-Thomas et al., 1986). These studies described the presence of enteric neurons in the gut, but the number of neurons found, highly depended on the experimental conditions used. Furthermore, in all of these studies melanocytes were found in the gut which were mainly present at the sites were ganglion formation would normally occur. In heterotopic quail-chick chimeras, in which the chicken vagal neural crest was replaced with quail neural crest from the adrenomedullary level (S18-24), quail cells were found along the entire digestive tract (LeDouarin and Teillet, 1974). But whereas in the preumbilical gut these quail cells had differentiated into enteric neurons, in the postumbilical gut these quail cells exclusively differentiated into melanocytes. In the reciprocal experiment, in which quail vagal neural crest was transplanted to the adrenomedullary level of a chicken embryo, they not only found quail cells in the normal adrenomedullary derivatives, but also in the postumbilical gut, providing further evidence for a specific interaction between vagal neural crest cells and the postumbilical gut regarding ENS formation. These experiments clearly indicate that, although neural crest cells from various axial levels can migrate through the gut and home to the correct sites, differentiation into enteric neurons, at least in the postumbilical gut, is confined

to vagal neural crest cells.

The vagal neural crest forms a transitional zone between the cranial and trunk neural crest. Cells from this region mainly emigrate from the neural tube between stages 9 and 12 and migrate along two principal pathways. Cells from the anterior vagal neural crest from the level of the mid-otic vesicle down to the third somite predominantly migrate along a dorsolateral pathway on their way to the three most caudal pharyngeal arches (Kuratani and Kirby, 1991; Miyagawa-Tomita et al., 1991). Ectomesenchymal derivatives of the vagal neural crest, such as the cardiac outflow tract and the mesenchymal components of the thymus and parathyroids, are exclusively derived from this part of the vagal neural crest (Kirby et al., 1983; Bockman and Kirby, 1984). Cells from the posterior vagal neural crest caudal to somite 2, migrate along a ventrolateral pathway through the rostral part of the somites (Bronner-Fraser et al., 1991; Miyagawa-Tomita et al., 1991). It has not been established along which migration pathway neural crest cells reach the gut. There is some indirect evidence that vagal neural crest cells migrate to the gut via the most caudal pharyngeal arches (Ciment and Weston, 1983; Payette et al., 1984; Tucker et al., 1986), but migration along a ventrolateral pathway has also been suggested (LeDouarin, 1982; Noden, 1988; Serbedzija et al., 1991).

It is generally agreed upon that neural crest cells enter the foregut at E2.5 (stage 16 or 17 HH) (LeDouarin and Teillet, 1973; Tucker et al., 1986), but the exact level of entry is hitherto unknown. These cells subsequently translocate in a craniocaudal direction, either through active migration or through passive displacement due to bowel elongation, following an exact time schedule (LeDouarin and Teillet, 1973; Tucker et al., 1986). There are few studies on the migration of neural crest cells through the gut. Tucker et al. (1986) described that neural crest cells migrate superficially, using the splanchnic epithelium as a substrate, whereas LeDouarin (1982) found that neural crest cells migrate as dispersed cells through the loose mesenchyme of the gut. Epstein et al. (1991) observed a complex network of neural crest-derived cells in the gut, indicating that neural crest cells interact with each other during migration. These studies, however, all agree upon the importance of the enteric mesenchyme in the guidance of neural crest cell migration, in the homing at specific sites and in the differentiation into the various types of enteric neurons. A number of molecules within the enteric mesenchyme have been described, which might play a role in one or more of these processes, i.e. fibronectin (Tucker et al., 1986), laminin (Pomeranz et al., 1991b), and HNK-1-carrying glycoproteins (Luider et al., 1992), but their relative importance has yet to be established.

2.5. Development of the enteric nervous system in mammals

Development of the mammalian ENS has been studied in human (Okamoto and Ueda, 1967),

and, more extensively, mouse embryos. Although ENS formation in mouse embryos is in principal the same as described for avian embryos, some apparent discrepancies should be mentioned here. Whereas in chicken embryos neural crest cells first enter the foregut and subsequently colonize the rest of the gut in a craniocaudal order, studies in mouse embryos suggested simultaneous colonization of fore- and hindgut at E9 (Rothman and Gershon, 1982; Gershon et al., 1984; Rothman et al., 1984; Rothman et al., 1986). Other studies, however, are in disagreement with these results and find a craniocaudally directed colonization process in mice as well (Nishijima et al., 1990; Kapur et al., 1992). A second difference lies in the fact that in mouse embryos transient catecholaminergic neurons are present in the wall of the gut, which are lost upon the arrival of sympathetic nerves (Rothman et al., 1987). In the avian gut, these transient catecholaminergic cells could only be observed in vitro, after dissociation of the gut and culturing of the enteric precursors outside the enteric microenvironment. These cells, however, were never found in vivo.

Much of the knowledge of mammalian ENS development comes from the study of animal models in which ENS formation is disturbed. Spontaneous occurrence of aganglionosis has been reported in mice, rats and horses. In mice, three different genetic mutations have been described, which all result in congenital megacolon combined with pigment abnormalities. The dominant spotting mutation is autosomal dominant. Heterozygotes are characterized by white-spotting and a deficiency of enteric neurons in the colon, whereas homozygotes die prior to gestational day 13 (Lane and Liu, 1984). Mutations in the autosomal recessive piebald-lethal (*Sl*) gene lead to a defect in the migration of cells from the neural crest (Webster, 1973). *Sl/Sl* homozygotes are white-coated except for patches of black pigment and invariably develop megacolon early in life (Lane, 1966). Animals often die from diarrhoea and enterocolitis before breeding age is attained, making the establishment of colonies very difficult. The lethal-spotted (*ls*) strain resembles the piebald-lethal, but homozygotes tend to survive longer making them more suitable for study (Lane, 1966). This gene is also transmitted in an autosomal recessive way.

In lethal-spotted mice, the distal 2 mm of the gut is aganglionic. Although this aganglionic segment lacks intrinsic innervation, it does receive many noradrenergic nerve fibers derived from neurons, whose cell bodies lie outside the gut (Payette et al., 1987). These extrinsic nerve fibers are characteristic of non-enteric peripheral nerves, because their axons are enveloped individually by Schwann cell processes with basal lamina and collagen-containing endoneurial sheaths (Tennyson et al., 1986c). The lack of intrinsic innervation in these mutant mice could be due to a defect in the neural crest or in the target organ. The occurrence of pigment abnormalities combined with ENS defects could point to a neural crest defect. In coculture experiments, however, neural crest cells from the foregut of *ls/ls* mice were found to be able to colonize the hindgut of both avians and control mice, whereas neither avian nor control mice crest was able to colonize *ls/ls* hindgut (Jacobs-Cohen et al.,

1987). Additional evidence that the primary defect in *ls/ls* mice is not neuroblast autonomous, comes from a study of Kapur et al., who made aggregation chimeras of cells derived from *ls/ls* mice and mice carrying the DßH-lacZ transgene (a marker for enteric neurons) (Kapur et al., 1993). They found that when more than 20% of the cells in chimeric mice were wild-type, the *ls/ls* phenotype was rescued. In addition, these rescued mice had mixtures of both *ls/ls* and wild-type neurons throughout the gut, including the distal rectum.

The hindgut of *ls/ls* mice shows an extraordinary overabundance and maldistribution of laminin and collagen type IV (Tennyson et al., 1986a; Tennyson et al., 1986b). These extracellular matrix molecules, which are normally found in basal lamina beneath the mucosal and serosal epithelia, and around smooth muscle cells and ganglia (Pomeranz et al., 1991b), are now present in a broad zone encompassing the entire mesenchyme of the aganglionic segment of the gut. This has led to the hypothesis that these basal lamina components may normally act as stop signals for neural crest cells, which carry specific receptors for these molecules (Pomeranz et al., 1991b), inducing them to cease migration, extend neurites and withdraw from the cell-cycle. Overabundance of these basal lamina components could then be interpreted as an extension of the normal mechanism in which neural crest cell migration is stopped prematurely, while not inhibiting axon growth.

Comparison of various animal species shows that the basic principles of ENS development are very similar. Each of these species, however, provides us with different tools to study the various aspects of ENS development.

2.6. Clinical disorders of the enteric nervous system

Study of spontaneous mutations might provide inside into normal embryonic development. An important congenital malformation involving the ENS in man, is congenital intestinal aganglionosis or Hirschsprung disease (HSCR). It is characterized by the absence of enteric ganglia in the most distal part of the bowel, combined with the presence of hypertrophic extrinsic nerve fibres. It was first described by Dr. Harald Hirschsprung (1830-1916), who described two newborns that presented with characteristic clinical features: severe defecation problems from birth onwards, increasing abdominal distension, enterocolitis with ulcers, a gradually declining general condition leading to death at the age of 7 and 11 month respectively. At first, the defect was thought to lie in the dilated and hypertrophied part of the colon, but Swenson was among the first to recognize that the primary defect lay in the distal non-dilatated colon (Swenson et al., 1949). In 1948, he introduced a new surgical technique, and since then children born with HSCR can be successfully operated, although some patients remain obstipated after surgery.

HSCR can be classified based on the length of the aganglionic segment, which always

encompasses the internal anal sphincter and sometimes extends well into the small bowel. Short-segment or classical HSCR is the best described congenital ENS malformation. The aganglionosis begins with the internal anal sphincter and extends proximally including the rectum and part of the sigmoid colon. In long-segment HSCR, aganglionosis extends beyond the sigmoid colon, sometimes involving the entire colon (total colonic aganglionosis or Zuelzer-Wilson disease), or even part of the small bowel. The longest aganglionic segment is found in total intestinal aganglionosis, a rare congenital malformation in which enteric neurons are lacking from the duodenum downwards to the anus. HSCR has an estimated overall population incidence of 1:5000 live-born children, a sex ratio of 3.9 to 1, males to females, and an overall risk to siblings of 4%.

Genetic study of HSCR has proven difficult, because of the very limited availability of large pedigrees. Until fairly recently, patients with HSCR hardly gave rise to offspring. Furthermore, having a child with HSCR often results in curtailment of child bearing. A positive family history has been described in approximately 7% of all cases. The high number of sporadic cases and the fact that the disease is four times more frequent in boys than in girls suggested a sex-modified multifactorial mode of inheritance, involving multiple genes (Passarge, 1983). Badner et al. (1990) performed complex segregation analysis on 487 probands and their families and showed that for short-segment HSCR the inheritance pattern was equally likely to be either multifactorial or due to a recessive gene with low penetrance. For long-segment HSCR, however, they found that the mode of inheritance was most compatible with an autosomal dominant gene with incomplete penetrance.

Deletions of the long arm of chromosome 10 (10q11) have been found in a patient with HSCR (Martucciello et al., 1992). Recently a gene for LS-HSCR was mapped to the proximal long arm of chromosome 10 (10q11.2) (Angrist et al., 1993; Lyonnet et al., 1993). Edery et al. (submitted for publication) described the *c-RET* proto-oncogene as the closest genetic marker with respect to the disease locus, suggesting that this proto-oncogene might be a candidate gene for HSCR. *c-RET* has also been shown to account for multiple endocrine neoplasia type 2A (MEN 2A), characterized by the occurrence of multiple neural crest-related tumors, such as medullary thyroid carcinomas and pheochromocytomas (Donis-Keller et al., 1993; Mulligan et al., 1993). The finding that transgenic mice carrying a null mutation of the *ret-1* gene have total intestinal aganglionosis, further suggests that the *RET-1* gene might be involved in the pathogenesis of long-segment HSCR (Dr. V. Pachnis, personal communication).

In mice, the *ret-1* gene maps to chromosome 14 where the piebald lethal gene has been localized (Badner et al., 1990). The existence of three different mouse models, however, each with a single gene defect, could point to the existence of more than one gene locus for HSCR (Lane, 1966). Identification of other possible candidate genes in humans comes from the study of chromosomal abnormalities associated with HSCR. Particularly striking is the

high incidence of HSCR with trisomy of chromosome 21, known as Down syndrome (Passarge, 1967). HSCR was also found to be associated with various deletions on the long arm of chromosome 13 (Sparkes et al., 1984; Lamont et al., 1989; Bottani et al., 1991). In these patients an association with craniofacial abnormalities and mental retardation has been described.

The various forms of HSCR are sometimes associated with other anomalies. For the long-segment ENS disorders associated anomalies have rarely been described, apart from occasional kidney abnormalities in total intestinal aganglionosis (DiLorenzo et al., 1985), and neuroblastomas in total colonic aganglionosis (Michna et al., 1988). For classical Hirschsprung disease the reported incidence of associated anomalies varies from 2.5 to 29.8% depending on the diligence with which they are sought and the manner in which they are reported (Graivier and Sieber, 1966; Swenson et al., 1973; Klein et al., 1984; Spouge and Baird, 1985; Ikeda and Goto, 1986; Ryan et al., 1992). Many of the associated anomalies are related to the third and fourth pharyngeal arches, receiving a contribution from the posterior rhombencephalic neural crest. These anomalies mainly entail heart defects, often characterized as tetralogy of Fallot (Lammer and Opitz, 1986; Larsen, 1993), whereas abnormalities of the thymus, which also receives a contribution from the posterior rhombencephalic neural crest, have never been reported (van Dommelen et al., chapter 3.7).

Next to the described forms of congenital aganglionosis, there are a number of other congenital disorders of the ENS worth mentioning here. Intestinal hyperganglionosis, or 'neuronal intestinal dysplasia', is characterized by an above-average number of neurons in the enteric ganglia and the presence of hyperplastic parasympathetic nerve trunks (Meier-Ruge, 1971; Howard and Garret, 1984). Colonic hypoganglionosis is characterized by a below-average number of enteric neurons and nerve fibers in the myenteric plexus (Meier-Ruge, 1971). Hypoganglionosis is described mostly in the transition zone between normal and aganglionic bowel of patients with HSCR (Gherardi, 1960; Walker et al., 1966; Garret et al., 1969; Meier-Ruge, 1969). There are a few reports of zonal aganglionosis (Tiffin et al., 1940; Haney et al., 1982; Tagushi et al., 1983; Seldenrijk et al., 1986), characterized by the presence of ganglionic segments within the aganglionic bowel, but its existence has been questioned (Yunis et al., 1983). Intestinal pseudo-obstruction is characterized by a functional obstruction of the ganglionic bowel and has often been associated with neuronal immaturity (Bughaighis and Emery, 1971; Erdohazi, 1974; Tanner et al., 1976). Besides congenital ENS disorders, there are also a number of acquired disorders of the ENS. In Chagas' disease enteric ganglia are lost secondary to a protozoan infection (Meneghelli, 1985). In patients with Parkinson's disease, ENS functioning can also be affected resulting in acquired megacolon (Kupsky et al., 1987). The finding of cytoplasmic hyaline inclusions in myenteric neurons which are identical to Lewy bodies found in the brain of Parkinson patients, further suggests a similarity between CNS and ENS neurons.

2.7. Conclusions

Study of the evolutionary origin of the ENS showed that ENS development preceded the origin of the vertebrate neural crest. In the moth, enteric neurons and glial cells arise from ectodermal placodes, in a way that shares several important features with the migratory behavior of vertebrate neural crest cells. The fact that no pharyngeal arch system has yet developed in the moth, indicates that ENS precursors do not have to migrate through the pharyngeal arches in order to be able to differentiate into enteric neurons.

Comparing various vertebrate species, we showed that, in general, basic principles of ENS development are very similar. Each species, however, offers certain advantages to study specific aspects of ENS development. Chicken embryos are well suited for experimental embryological manipulations, and in this species specific markers are available both for neural crest cells and for enteric neurons. Genetic study of intestinal aganglionosis can be performed in mammalian embryos. In mice, three spontaneously occurring mutants led to the identification of three recessive genes involved in ENS development. Recently described markers for enteric neural crest cell precursors and enteric neurons in mice, could further facilitate the study of the defects in ENS development occurring in each of these mutants. In humans, search for chromosomal abnormalities and linkage analysis in large pedigrees of HSCR patients already led to the identification of the *c-RET* proto-oncogene as a candidate gene for HSCR.

2.8. References

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Chapter 3

The experimental work

3.1. Introduction to the experimental work

In this thesis, we studied the development of the enteric nervous system and the specific role of the posterior rhombencephalic neural crest in this process. We used the avian embryo as a model system, because it is easily amenable to experimental manipulation and we found in our species comparison (chapters 1 and 2) that the basic principles of neural crest cell migration and ENS development are very similar in birds and mammals. The experimental work described in this thesis is related to three specific questions. 1) Is the neural crest regionally specified with regard to ENS formation? 2) Which cells or tissues provide homing and/or differentiation signals for neural crest cells in the gut? 3) What do experiments of nature teach us about ENS development?

It is generally agreed upon that the ENS is derived from the vagal neural crest adjacent to the first seven somites (reviewed in LeDouarin, 1982). For the most part, this vagal neural crest lies at the level of the posterior rhombencephalon, from the level of the otic vesicle down to the caudal boundary of somite 5. We studied, if there is a specific axial segment within the posterior rhombencephalic neural crest which is primarily responsible for ENS formation. The ablation experiments, described in chapter 3.2., showed that the neural crest adjacent to somites 3-5 is essential for ENS formation in the colon in vivo. We found, however, that vagal neural crest segments anterior to somite 3 were also capable of ENS formation in the hindgut. Trunk neural crest cells, although able to colonize the gut, did not give rise to enteric neurons or ganglia and instead differentiated into melanocytes (chapter 3.2. and 3.5.). We also found that vagal neural crest cells which had been in prolonged contact with the neural tube had an increased capability to form enteric ganglia, suggesting that precursors for enteric neurons either emigrate later than hitherto assumed or require close contact with the neural tube for a prolonged time period in order to be able to differentiate into enteric neurons. Furthermore, we found that vagal neural crest cells, cultured for one day in vitro were still capable of forming enteric ganglia, whereas after four days of culture they had lost this capacity and differentiated into melanocytes, suggesting that vagal neural crest loose their specific characteristics upon culture and start behaving similar to trunk neural crest cells.

It has been established that the ectomesenchymal derivatives of the posterior rhombencephalic crest are sensitive to retinoic acid (RA) both in humans (Lammer et al., 1985) and in a number of animal species (Shenefelt, 1972; Fantel et al., 1977; Kamm, 1982; Webster et al., 1986), but there have been no reports on an adverse effect of RA on ENS development. Both in the posterior rhombencephalic crest and in enteric ganglia, however, RA receptors and binding proteins have been found. Therefore, we studied the effect of RA administration in vivo at various developmental stages, in a dose known to affect the ectomesenchymal derivatives, but we found no disturbances in ENS formation. These results

give further evidence for the existence of distinct subpopulations of neural crest cells for the various derivatives of the posterior rhombencephalic neural crest (chapter 3.3.).

Secondly, we studied the role of the enteric microenvironment in neural crest cell colonization. We found that neural crest cells which had already colonized the gut were attracted by aneural gut, whereas neural gut was not capable of attracting neural crest cells (chapter 3.4.). We then studied the microenvironment of aneural gut in order to identify cell types which could be involved in neural crest cell homing and/or differentiation. We found a layer of mesenchymal cells within the submucosa of aneural gut which reacted with the monoclonal antibody HNK-1, a pattern of immunoreactivity which we called HNK-1 mode 1. We studied the expression of the HNK-1 epitope, because it was found to be present on migrating neural crest cells (Vincent et al., 1983) and thought to be involved in cell adhesion (Kruse et al., 1984), and because it was also found to be expressed by other tissues at the time they are developmentally active (Stern and Canning, 1990). Study of the origin of these HNK-1 immunoreactive mesenchymal cells showed that these cells arise through de-epithelialization from the splanchnic epithelium, both in the presence and absence of any form of extrinsic innervation (Souren et al., unpublished).

Biochemical characterization of the HNK-1 immunoreactive mesenchymal cells, which constitute approximately 10% of the total amount of cells, identified two HNK-1 carrying cell membrane glycoproteins of 42 and 44 kD (chapter 3.6.). The HNK-1 mode 1 immunoreactivity in aneural gut disappeared when vagal neural crest cells colonized the gut and formed enteric ganglia (chapter 3.5.). When trunk neural crest cells colonized the hindgut, they differentiated into melanocytes and HNK-1 mode 1 persisted.

Most of the information on ENS development, described in this thesis so far, has been obtained through experimental embryology mainly using avian or mouse embryos. Study of human embryology, especially those cases in which something went wrong (experiments of nature), could provide valuable additional information on ENS development. We performed a retrospective clinical study of patients with ENS malformations and investigated the occurrence of associated anomalies. The results of this study, in which we also looked for minor abnormalities and dysmorphic signs, are described in chapter 3.7. They show that the percentage of associated anomalies occurring with the various ENS malformations (classified according to the lengths of the aganglionic segment), are higher than reported thus far. Furthermore, we found that with an increasing lengths of the aganglionic segment, the percentage of associated anomalies increased. In the majority of SS-HSCR and LS-HSCR cases in humans, aganglionosis seems to be an isolated defect (77.1% and 54.5% respectively). We classified the remaining SS- and LS-HSCR patients into four groups depending on the character of their associated anomalies. In the first group, SS-HSCR was associated with Down syndrome (7.6%). These patients were predominantly males. The second group entailed 'syndromic' cases of HSCR the incidence of which was highest in LS-

HSCR (20.5% versus 5.9% in SS-HSCR). The sex ratio in 'syndromic' cases was 1 : 1 in both SS- and LS-HSCR. In the third group, HSCR was associated with craniofacial dysmorphisms, whereas the fourth group entailed HSCR patients with one or more anatomic abnormalities. Such a subdivision of patient groups could help considerably in the search for the underlying, possibly genetic, defect in Hirschsprung disease.

Ablation of Various Regions Within the Avian Vagal Neural Crest Has Differential Effects on Ganglion Formation in the Fore-, Mid- and Hindgut

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ABSTRACT The vagal neural crest adjacent to the first seven somites gives rise to both ganglionic and ectomesenchymal derivatives. Ganglionic derivatives are the neurons and supportive cells of the enteric nervous system (ENS), cardiac, and dorsal root ganglia. Ectomesenchymal derivatives are cells in the cardiac outflow tract and the mesenchymal components of thymus and parathyroids. Ectomesenchymal derivatives are formed by a segment of the vagal neural crest, from the level of the otic vesicle down to the caudal boundary of the third somite, called the cardiac neural crest. We performed neural crest ablations to study regional differences within the avian vagal neural crest with regard to the formation of the ENS. Ablation of the entire vagal neural crest from the mid-otic vesicle down to the seventh somite plus the nodose placode resulted in the absence of ganglia in the midgut (jejunum and ileum) and hindgut (colon). The foregut (esophagus, proventriculus, gizzard, and duodenum) was normally innervated. After ablation of the vagal neural crest adjacent to somites 3–5, ganglia were absent in the hindgut. Ablations of vagal neural crest not including this segment had no effect on the formation of the ENS. We surmise that the innervation of the hindgut in vivo depends specifically on the neural crest adjacent to somites 3–5, whereas innervation of the midgut can be accomplished by all segments within the vagal neural crest. The foregut can also be innervated by a source outside the vagal neural crest.

To study intrinsic differences between various vagal neural crest segments regarding ENS formation, we performed chorioallantoic membrane cocultures of segments of quail vagal neural anlage and E4 chicken hindgut. We found that all vagal neural crest segments were able to give rise to enteric ganglia in the hindgut. When the neural crest of somites 6 and 7 was included in the segment, we also found melanocytes in the hindgut, suggesting that this segment is more related to trunk neural crest. Furthermore, we found that the vagal neural anlage from older embryos (>18 somites) showed an increased potential to form enteric ganglia. This suggests that vagal neural crest cells that have been in prolonged contact with the neural tube in vivo, because of either late emigration or delayed migration, have an increased probability to form enteric ganglia.

Key words: Neural Crest, Ablation, HNK-1, Enteric nervous system, Melanocytes, Rhombomeres, Segmentation

INTRODUCTION

Segmentation is a widely employed strategy in development. In the vertebrate head, the most prominent manifestation of segmentation is found in the hindbrain, where the cranial neural crest is associated with segmental units in the central nervous system called rhombomeres (Lumsden and Keynes, 1989; Keynes et al., 1990; Guthrie and Lumsden, 1991; Lumsden et al., 1991). The migration pathways and developmental fate of neural crest cells in the hindbrain have been studied in isotopic quail-chick chimeras (Le Lièvre and Le Douarin, 1975), by grafting of cells labelled with tritiated thymidine (Noden, 1975), and more recently by microinjection of the fluorescent dye DiI (Lumsden et al., 1991). In this way it has been established that the neural crest of a certain rhombomere migrates to a particular pharyngeal arch to form its specific derivatives. Noden (1983) showed that the anterior rhombencephalic neural crest is already committed to a certain phenotype before the onset of migration. He transplanted neural crest associated with rhombomeres 1 and 2, which will normally populate the first pharyngeal arch, to the second pharyngeal arch area associated with rhombomeres 3 and 4, and found that this led

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to the formation of first arch structures within the second arch. In addition, he showed that this cephalic neural crest has patterning activity, because the ectopic mandibles had a set of muscles attached to them that were derived from the second arch but resembled first arch muscles (Noden, 1988).

Recently, it was found that the cranial neural crest is not only regionally, but also temporally specified. Cranial neural crest cells emerging at later times have an increased probability of assuming a ganglionic rather than an ectomesenchymal fate (Lumsden et al., 1991). Temporal specification was also described for trunk neural crest (Artinger and Bronner-Fraser, 1992). It was shown that late-emigrating trunk neural crest cells are partially restricted in their developmental potential and mainly differentiate into melanocytes while no longer capable of giving rise to adrenergic neurons. There is, however, no clear evidence for regional specification of the trunk neural crest. Here migration of the neural crest is largely determined by the paraxial mesoderm. Neural crest cells emerge from the neural tube in an unsegmented way and are subsequently restricted to the anterior part of the somite (Rickmann et al., 1985; Teillet et al., 1987). This segmented migration correlates with intrinsic differences between the anterior and posterior parts of the somites (Keynes and Stern, 1984; Stern and Keynes, 1987; Kalcheim and Teillet, 1989).

The vagal neural crest adjacent to the first seven somites, forms a transitional zone between cranial and trunk neural crest. The vagal neural crest is generally considered to be the source for the neurons and supportive cells of the ENS along the entire digestive tract (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Allan and Newgreen, 1980). Apart from this contribution to the ENS, the vagal neural crest also gives rise to cardiac and dorsal root ganglia and ectomesenchymal derivatives. Dorsal root ganglia are formed by the neural crest caudal to somite 5. Ectomesenchymal derivatives, such as cells in the cardiac outflow tract (Kirby et al., 1983), thymic stromal cells (Bockman and Kirby, 1984), and the mesenchymal component of the parathyroids (Le Lièvre and Le Douarin, 1975), and the cardiac ganglia (Kirby and Stewart, 1983) are formed by the cardiac crest from the level of the otic vesicle down to the caudal boundary of the third somite. It has been shown that there is regional specification within this cardiac neural crest (Kirby et al., 1985; Besson et al., 1986). Using ablation experiments, Besson et al. showed that the size and the location of the lesions influenced both the incidence and the type of cardiac defects. Formation of the cardiac ganglia after ablation of the cardiac neural crest could be partially rescued by the nodose placode, which proved to be capable of giving rise to the neuronal derivatives of the cardiac crest (Kirby, 1988).

We studied regional differences within the vagal neural crest with regard to the formation of the ENS using two experimental systems. First, we performed neural crest ablation experiments to study regional differences in vivo. In a second set of experiments, we cocultured different segments of quail vagal neural crest and aneural chicken hindgut, on the chorioallantoic membrane to study intrinsic differences between these vagal neural crest segments. Using this coculture system, we also studied whether the vagal neural crest is temporally specified with regard to the formation of ectomesenchymal and ganglionic derivatives.

TABLE 1. Formation of Enteric Ganglia After Neural Crest Ablation[a]

Ablation	n	Foregut	Midgut	Hindgut
MO-S7 + placode	6	+	−	−
MO-S3 ± placode	5	+	+	+
S1	3	+	+	+
S1–2	3	+	+	+
S3–5	8	+	+	−
S3–7	3	+	+	+ (1) − (2)
S6–7	6	+	+	+

[a]The different parts of the gut were analyzed for the presence of enteric ganglia using the HNK-1 antibody. + indicates normal innervation; − indicates absence of enteric ganglia. The number between parentheses indicates the number of embryos with or without enteric ganglia. Absence of such a number indicates that all embryos within a group gave identical results.

RESULTS

Neural Crest Albations and the Development of the Enteric Nervous System

Normal neural crest cell colonization of the chicken gut occurs between stage 19 and 32 (E3.5-E8) (Meijers et al., 1987). We performed different types of neural crest ablations (listed in Table 1) at stage 8, and studied the presence of enteric ganglia at E11, a stage at which ganglion formation is normally completed. Ablations including the entire vagal neural crest from the level of the mid-otic vesicle (at the boundary between rhombomeres 5 and 6) down to the posterior boundary of somite 7 together with the nodose placode, were expected to result in aganglionosis, defined as absence of enteric ganglia, in the entire gut. The nodose placode, known to be a compensatory source for cardiac ganglia after ablation of the cardiac crest, was included in the ablation to exclude possible compensation for the enteric ganglia. We found that the esophagus, proventriculus, gizzard, and duodenum contained enteric ganglia. In the duodenum, enteric ganglia were observed on each side of the circular smooth muscle layer (Fig. 1). Outside the muscle layer the prominent myenteric plexus was present, whereas the submucous plexus was less well developed. Staining with hematoxylin clearly showed the presence of neurons within the enteric ganglia characterized as large cells with a large nucleus and a clear nucleolus (Fig. 1A,B). Both plexuses were strongly immunoreactive with the monoclonal antibodies HNK-1 and RMO 270. HNK-1 stained perikarya

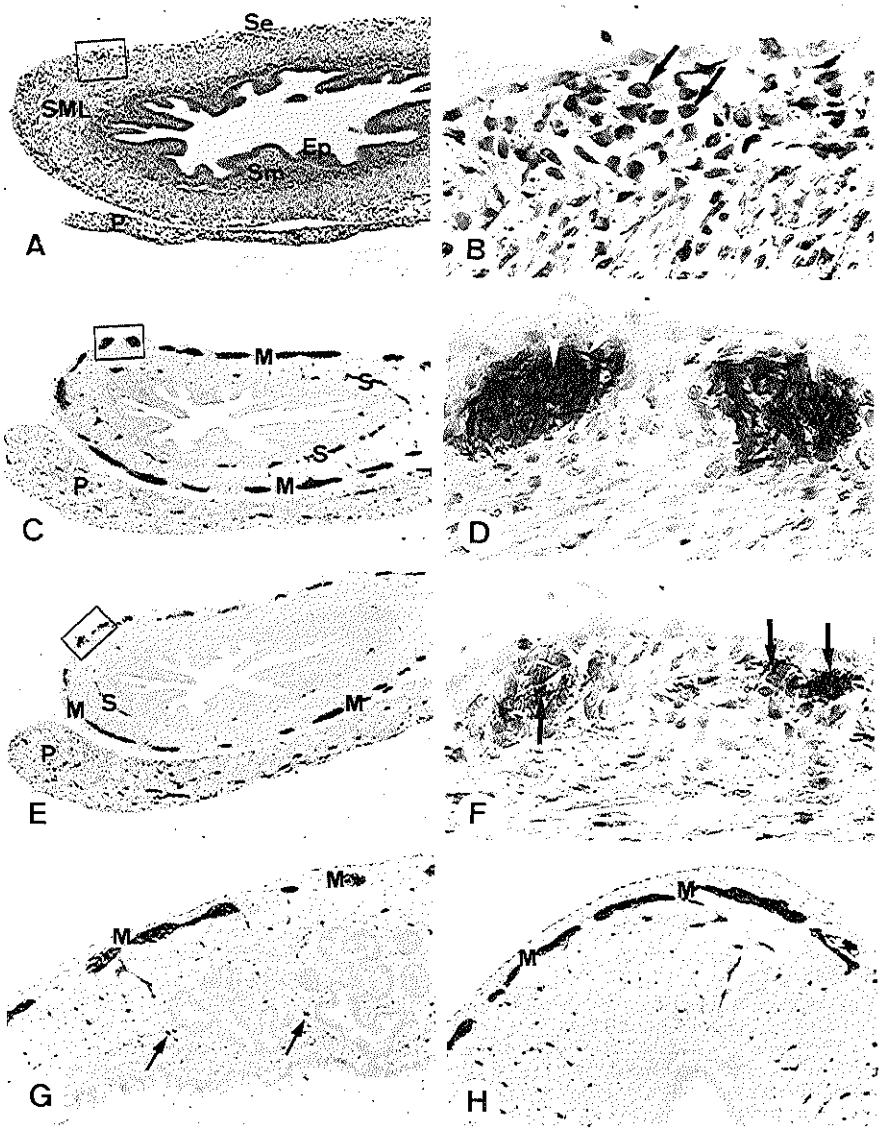

Fig. 1. Paraffin sections of E11 chicken gut after ablation the vagal neural crest from MO-S7 at stage 10. A–F: Duodenum. **A:** Hematoxylin staining showing the different layers in the duodenum. Ep: epithelium, Sm: submucosa, SML: smooth muscle layer, So: serosa. P = pancreas. ×16. **B:** Detail of A, showing myenteric ganglia containing neurons (arrows). ×63. **C:** Immunoperoxidase staining with HNK-1, showing the myenteric (M) and submucous (S) ganglia. ×16. **D:** Detail of C, showing both neurons (arrowheads) and extrinsic nerve fibres (arrows) within the myenteric ganglia. ×63. **E:** Immunoperoxidase staining with RMO 270 clearly showing myenteric (M) and, to a lesser extent submucous ganglia (S). ×16. **F:** Detail of E, showing extrinsic nerve fibres (arrows) within the myenteric ganglia. ×63. **G:** Oesophagus and proventriculus: the HNK-1 antibody visualizes myenteric ganglia (M) and dispersed cells in the submucosa (arrows). ×16. **H:** Gizzard: the HNK-1 antibody shows myenteric ganglia (M) and dispersed staining of cells and fibres in the muscle layer and the submucosa. ×16.

and intrinsic and extrinsic nerve fibres within the enteric ganglia (Fig. 1C,D), whereas RMO 270 mainly stained nerve fibres (Fig. 1E,F). In the esophagus, proventriculus and gizzard staining with the monoclonal antibody HNK-1 showed prominent myenteric ganglia and dispersed immunoreactive cells within the submucosa (Fig. 1G,H). We found that the gut distal to the duodenum was aganglionic. Outside the muscle layer plexus-like structures were present, but staining with hematoxylin showed that these plexuses did not contain neurons (Fig. 2A,B). Staining with the HNK-1 antibody (Fig. 2C,D) revealed a layer of HNK-1 immunoreactive mesenchymal cells in the submucosa and staining at the site of the myenteric plexus previously seen in cultures of E4 hindgut (Luider et al., 1992). Staining with RMO 270 (Fig. 2E,F) showed the presence of extrinsic nerve fibres within the myenteric plexus, but not at the site of the submucous plexus.

After ablation of the neural crest from the level of the mid-otic vesicle down to the posterior boundary of somite 3 (MO-S3), the entire gut contained enteric ganglia (Fig. 3A,B). Including the nodose placode into the ablation did not influence this result, indicating that the nodose placode did not function as a compensatory source. Ablation of the neural crest at the level of somites 3–5 or 3–7 led to aganglionosis of the colon in 10 out of 11 embryos studied (Fig. 3C,D), even in the presence of the nodose placode. In these 10 embryos there was a sharp boundary between the ganglionic and aganglionic part of the gut situated at the level of the ceca. The ceca were normally innervated, whereas the colon was aganglionic. Ablation of the neural crest adjacent to somites 6–7 had no effect on the innervation of the gut (Fig. 3E,F).

The results from these ablation experiments, summarized in Figure 4, show that the neural crest adjacent to somites 3–5 is essential for the formation of enteric ganglia in the hindgut, whereas the neural crest from MO to S3 and S6 to 7 is not essential for ENS formation. Furthermore, these results show that the foregut can be innervated by a source outside the vagal neural crest.

Colonization Assay

All parts of the vagal neural crest are capable of forming enteric ganglia in the hindgut. We studied whether the results obtained in our ablation experiments were based on intrinsic differences between the various neural crest segments regarding their ability to innervate the hindgut. The vagal neural anlage from quail embryos having 22 to 28 somites (stages 15–16) was divided into small segments and cocultured with chicken aneural hindgut on the chorioallantoic membrane. In Table 2 the various vagal neural crest segments used in this coculture system are listed. All the segments tested were able to give rise to a normal pattern of enteric ganglia in the hindgut (Fig. 5). Staining with Hoechst 33258 confirmed that the cells within the enteric ganglia were of quail origin. These results indicate that an amount of vagal neural crest equivalent to the lengths of two somites is sufficient for colonization of the hindgut. In 2 out of the six cocultures in which the posterior part of the vagal neural crest at the level of somites 4–7 was included, melanocytes were present in the hindgut (Fig. 5D).

We conclude that with this coculture system a regional specification could be demonstrated regarding the neural crest adjacent to somites 6–7. While all vagal neural crest segments were able to give rise to enteric neurons, neural crest segments including somites 6–7 in addition gave rise to melanocytes in the hindgut.

Vagal neural crest cells from embryos of stages 13–16 show an increased potential to form enteric ganglia. To study temporal specification within the vagal neural crest, we studied whether vagal neural crest cells from embryos of various developmental stages were equally capable of forming enteric ganglia in the hindgut. We explanted vagal neural anlagen from embryos having 9 to 28 somites (stages 10–16) and tested these in our coculture system. In 9 out of 10 cocultures in which the vagal neural crest was taken from an embryo with 20 to 28 somites, a normal pattern of enteric ganglia was observed (Fig. 6A). Of the 10 cocultures with vagal neural crest from embryos with 18 or less somites, only 1 showed a normal amount of enteric ganglia. In 5 cocultures with neural crest from younger embryos, enteric ganglia were present, but these were smaller, containing fewer enteric neurons, and present in less abundance (Fig. 6B). In 4 of these cocultures no enteric ganglia were present. The results are listed in Table 3.

We conclude that, in our coculture system, vagal neural crest cells from older embryos, that have been in prolonged contact with the neural tube in vivo, have an increased potential to form enteric ganglia compared to vagal neural crest cells from younger embryos.

DISCUSSION

Regional Differences Within the Vagal Neural Crest Regarding ENS Formation

We investigated whether there are regional differences within the vagal neural crest with regard to the formation of the ENS using two microsurgical approaches. We found that ablation of the entire vagal neural crest from the otic vesicle down to the seventh somite resulted in aganglionosis of the mid- and hindgut. The foregut down to the level of the duodenum was normally innervated. Ablation of the neural crest from the mid-otic vesicle down to somite 3 had no effect on enteric ganglia formation in the entire gut, whereas ablation of the neural crest of somites 3–5 resulted in aganglionosis of the hindgut. These results indicate that the dependence on specific neural crest segments differs for the various parts of the gut. Innervation of the hindgut depends on a specific segment of the vagal neural crest adjacent to somites 3–5. Innervation of the

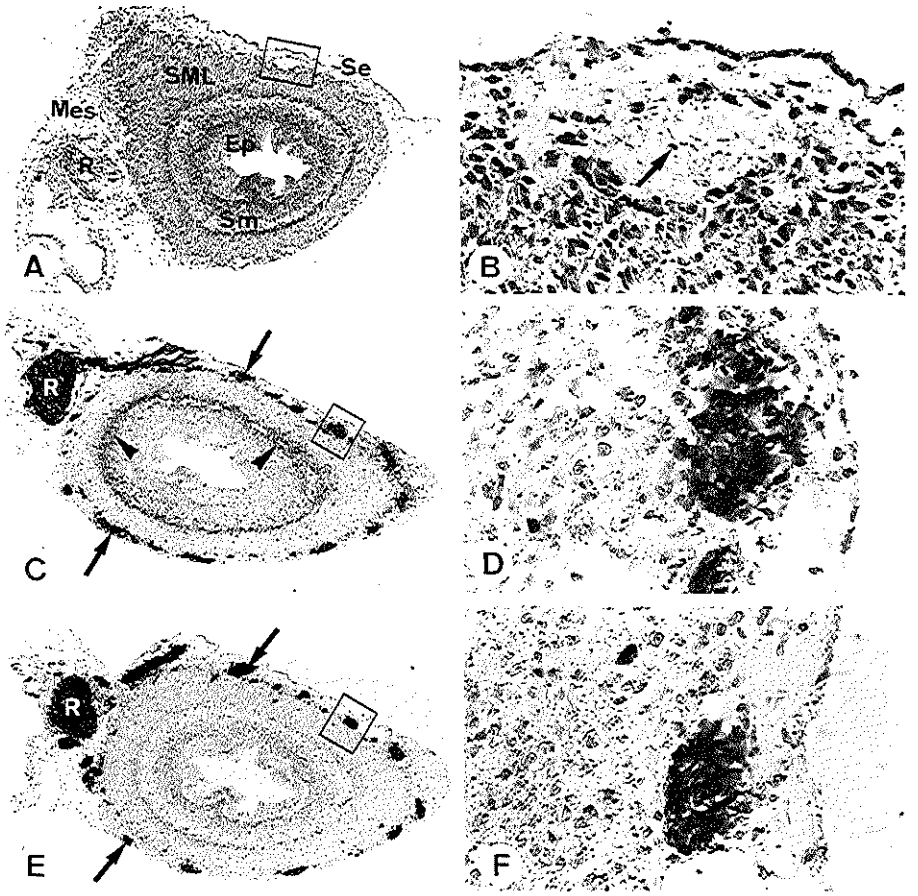

Fig. 2. Paraffin sections of E11 chicken colon after ablation of the vagal neural crest at the level MO-S7 at stage 10. **A:** hematoxylin staining showing the different layers in the colon. Ep: epithelium, Sm: submucosa, SML: smooth muscle layer, Se: serosa, Mes: mesentery, R: ganglion of Remak. × 16. **B:** Detail of A, showing the neuron-free myenteric plexus. Note the presence of one cell with a small nucleus, not characteristic for neurons within the plexus (arrow) ×63. **C:** Immunoperoxidase staining with the HNK-1 antibody showing staining at the site of the myenteric plexus (arrows), plus an additional band of HNK-1 immunoreactive mesenchymal cells in the submucosa (arrowheads). Remak's ganglion (R) is also stained. × 16. **D:** Detail of C, showing a myenteric plexus containing extrinsic nerve fibres and no neurons. ×63. **E:** The RMO 270 antibody shows immunoreactivity at the site of the myenteric plexus (arrows), but not in the submucosa. Remak's ganglion (R) is also stained. ×16. **F:** Detail of E, showing a myenteric plexus containing extrinsic nerve fibres. ×63.

midgut, although dependent on the presence of vagal neural crest, does not depend on a specific segment. The foregut, which is normally innervated by the vagal neural crest, can also be innervated by a source outside the vagal neural crest. This might be related to intrinsic differences between the various vagal neural crest segments in their ability to innervate different parts of the gut. This could, however, also be caused by a difference in the extend of compensatory mechanisms for the various parts of the gut.

McKee and Ferguson (1984) performed unilateral or bilateral extirpation of the mesencephalic neural crest in chicken embryos and found that the mesencephalic region was repopulated by 'new' neural crest cells migrating from adjacent anterior or posterior neuraxial levels. Outflow septation of the heart depends specifi-

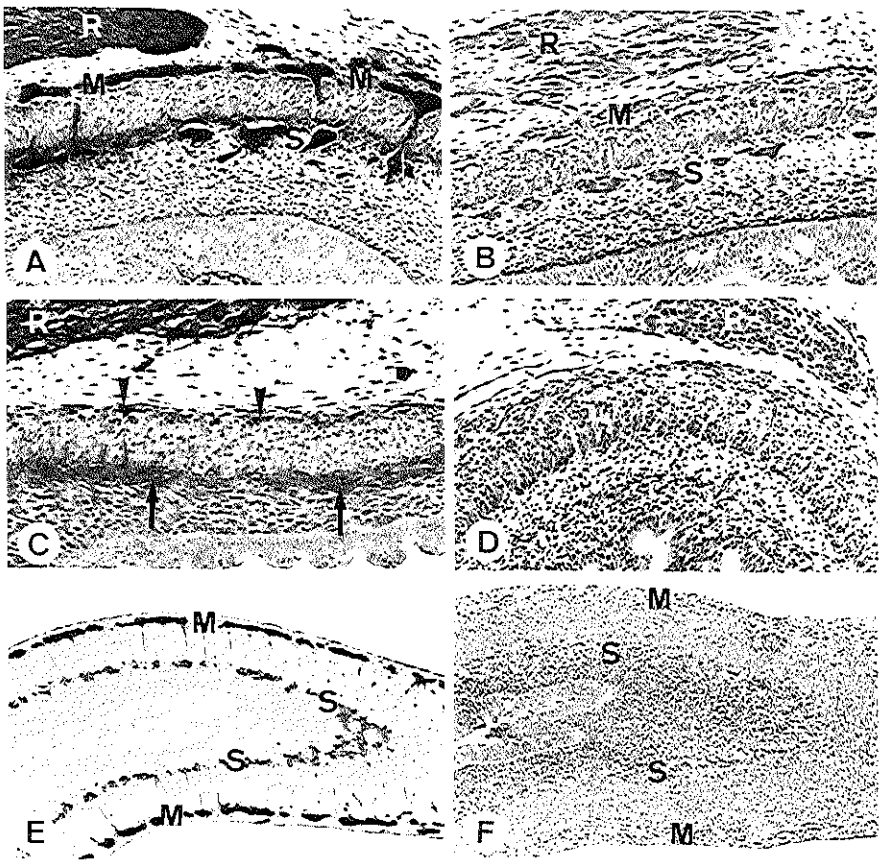

Fig. 3. Paraffin sections of E12 colon after ablation of part of the vagal neural crest at stage 10. A: Ablation at the level of the otic vesicle down to the caudal boundary of somite 3; HNK-1 immunoperoxidase staining showing the myenteric (M) and submucous (S) ganglia. Remak's ganglion (R) is also stained. ×40. B: Same section as in A stained with hematoxylin showing enteric ganglia. ×40. C: Ablation at the level of somites 3–5; HNK-1 immunoperoxidase staining showing a band of mesenchymal cells in the submucosa (arrows) and staining at the site of the myenteric plexus (arrowheads). Remak's ganglion (R) is also stained. ×40. D: Similar section as in C; staining with hematoxylin shows the absence of enteric ganglia. ×40. E: Ablation at the level of somites 6–7; HNK-1 immunoperoxidase staining shows the presence of myenteric (M) and submucous (S) ganglia. ×40. F: Similar section as in E, stained with hematoxylin. ×40.

cally on the cardiac neural crest and can not be compensated by more anterior or posterior crest (Kirby et al., 1983, 1985; Besson et al., 1986). Cardiac ganglia, however, which also derive from the cardiac crest, can be formed by cells derived from the nodose placode (Kirby, 1988). These results suggest that compensatory mechanisms might vary for the different segments of the neural crest and may also depend on the specific derivatives of each segment. We found normal innervation of the foregut after ablation of the entire vagal neural crest. This could mean that the vagal neural crest, which gives rise to enteric ganglia along the entire digestive tract in quail-chick chimeras (Le Douarin and Teillet, 1973), may not be the only source for enteric ganglia in the foregut. Yntema and Hammond (1954) described aganglionosis of the entire digestive tract after ablation of the vagal neural crest. This was only the case, however, when the ablation included the anterior rhombencephalic neural crest from the otic vesicle up to the level of the fifth cranial nerve (corre-

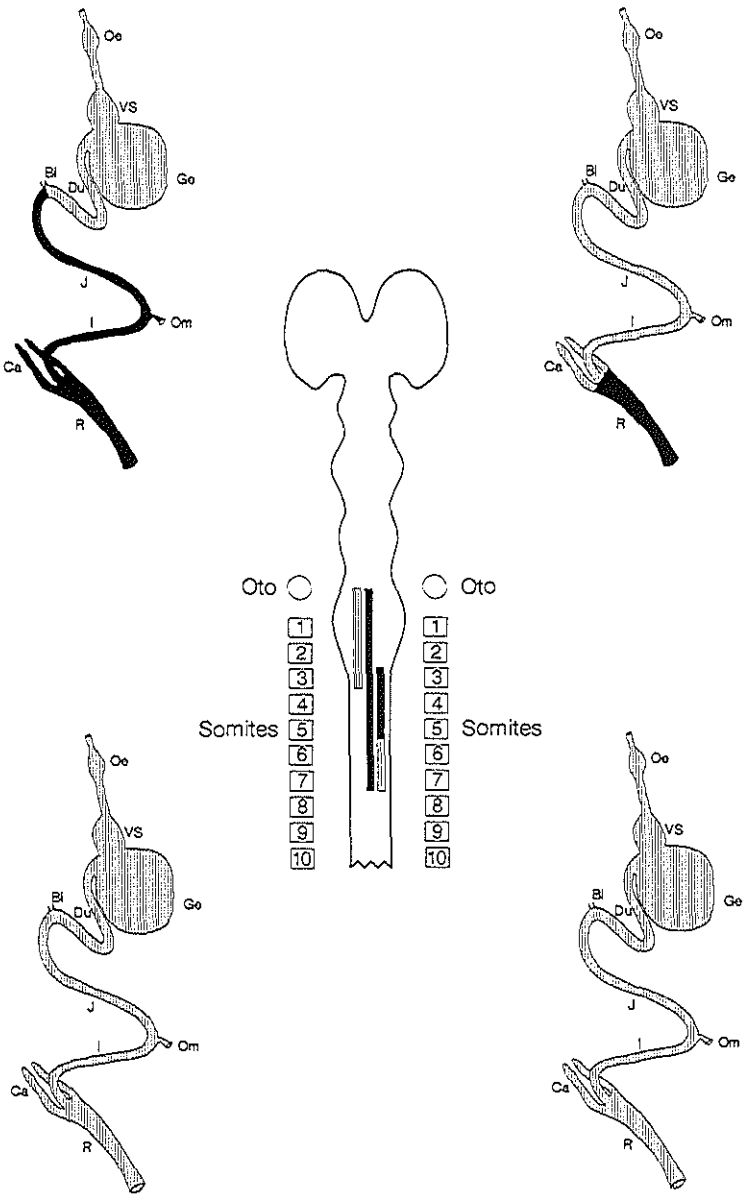

Fig. 4. Schematic drawing summarizing the results of the various neural crest ablations. The bars in the embryo indicate the ablated segment of the neural crest. The parts of the gut coloured black indicate the extent of the aganglionosis, the hatched parts are normally innervated. **Top left:** ablation of MO-S7; **top right:** ablation of S3–5; **bottom left:** ablation MO-S3; **bottom right:** ablation S6–7. Oe: oesophagus, Vs: proventriculus, Ge: gizzard, Du: duodenum, Bl: bile duct, J: jejunum, Om: umbilicus, I: ileum, Ca: ceca, R: rectum, Oto: otic vesicle.

TABLE 2. Colonization Assay With Segments of the Vagal Neural Crest[a]

Segment of crest	n	Enteric ganglia	Melanocytes
S1–3	5	4	0
S4–5	3	2	0
S4–7	2	2	1
S6–8	4	4	1

[a]The different segments of vagal neural crest are indicated by the numbers of the adjacent somites. n indicates the number of cocultures performed within each group. Enteric ganglia were visualized with the HNK-1 antibody. The amount of cocultures showing enteric ganglia and/or melanocytes are indicated.

sponding to rhombomere 2). When the ablation included only the posterior rhombencephalic crest from the otic vesicle caudad, they too found enteric neurons in the foregut. This indicates that, in the absence of the vagal neural crest, the anterior rhombencephalic neural crest may also be capable of giving rise to enteric neurons in the foregut. Our finding that ablation of the cardiac crest does not result in disturbed ENS formation, could be due to compensation by neural crest anterior to the otic vesicle or posterior to somite 3. Another explanation could be that this part of the vagal neural crest does not contribute to ENS formation in vivo. We performed isotopic quail-chick chimeras and found that the neural crest of somites 3–5 gave rise to enteric ganglia along the entire gut. In chimeras of the neural crest of MO–S2, we did not find quail cells in the gut, except for one chimera in which few quail cells were present in the fore- and midgut. In all chimeras of the neural crest of MO–S2, however, quail cells were found in the heart (unpublished results). In a similar study, Le Douarin and Teillet (1973) constructed isotopic quail-chick chimeras, containing various parts of the vagal neural crest. Chimeras containing the neural crest of somites 1–6 or somites 4–9 both gave rise to an ENS consisting almost entirely of quail cells. Chimeras of the neural crest of somites 6–13 gave rise to an ENS consisting of both chicken and quail cells. The results of these various types of chimeras, combined with the data of our chimeras and ablations, strongly suggest the importance of the neural crest of somites 4 and 5 for ENS formation.

After ablation of the neural crest of somites 3–5 enteric ganglia were absent in the hindgut, indicating that neural crest cells anterior and/or posterior to this segment can give rise to enteric ganglia in the fore- and midgut, but not in the hindgut. We always found a sharp boundary between the aganglionic and the ganglionic part of the gut, which was situated at the level of the ceca. Such a sharp boundary makes it less likely that aganglionosis in the hindgut is caused by a mere quantitative defect, that is a shortage of enteric precursors following ablation. Our results indicate that innervation of the hindgut in vivo depends specifically on the neural crest adjacent to somites 3–5, and can not be compensated by other sources.

Regional Differences Within the Vagal Neural Crest May Be Related to Migration Pathways

When we performed cocultures of small segments of quail vagal neural anlage and E4 chicken hindgut, we found that all different segments tested were capable of forming normal enteric ganglia. In a previous study (Peters-van der Sanden et al., 1993), we used the same coculture system and demonstrated an intrinsic difference between vagal and trunk neural crest cells in their ability to innervate the hindgut. Although both vagal and trunk neural crest cells were able to colonize the hindgut, vagal neural crest cells differentiated into enteric neurons, whereas trunk neural crest cells mainly differentiated into melanocytes. Since we were not able to demonstrate intrinsic differences between various vagal neural crest segments, the special features of the neural crest at the level of somites 3–5 observed in the ablation experiments must be ascribed to an in vivo process, that does not take place in the coculture system. An important difference in the coculture system compared to the in vivo situation, is the direct association of the neural anlage and the gut, bypassing the normal migration pathways in the embryo. The migration pathways of anterior rhombencephalic and cardiac neural crest cells have been the subject of extensive investigations (Noden, 1975, 1983; Kuratani and Kirby, 1991; Lumsden et al., 1991; Miyagawa-Tomita et al., 1991), but migration of the posterior vagal neural crest cells has been less well studied. Recently, using whole-mount staining with the HNK-1 antibody, it has been established that cardiac neural crest cells migrate predominantly along a dorsolateral pathway on their way to the third, fourth, and sixth pharyngeal arches (Kuratani and Kirby, 1991). These crest cells form the circumpharyngeal crest (Kuratani and Kirby, 1991; Miyagawa-Tomita et al., 1991), a compact population of neural crest cells which is formed at stage 11 and gives rise to the pharyngeal ectomesenchyme. Caudad to the second somite, part of the crest cell population migrates along a ventrolateral pathway through the rostral part of the somites (Rickmann et al., 1985; Teillet et al., 1987). Caudad to the third somite this pathway becomes the predominant one (Bronner-Fraser, 1986). It was found that the neural crest cells adjacent to somites 4–7 are the most anterior neural crest cells that do not populate the pharyngeal arches (Miyagawa-Tomita et al., 1991). This could mean that the neural crest cells that are essential for the innervation of the hindgut and that could perhaps be responsible for the innervation of the entire gut, migrate along a pathway that differs from the one followed by anterior vagal neural crest cells.

In the present study we found that, in cocultures, the neural crest adjacent to somites 6–7, besides giving rise to enteric ganglia, also led to the formation of occasional melanocytes in the gut. Previous coculture experiments showed that trunk neural crest cultured with aneuronal hindgut gives rise to melanocytes in

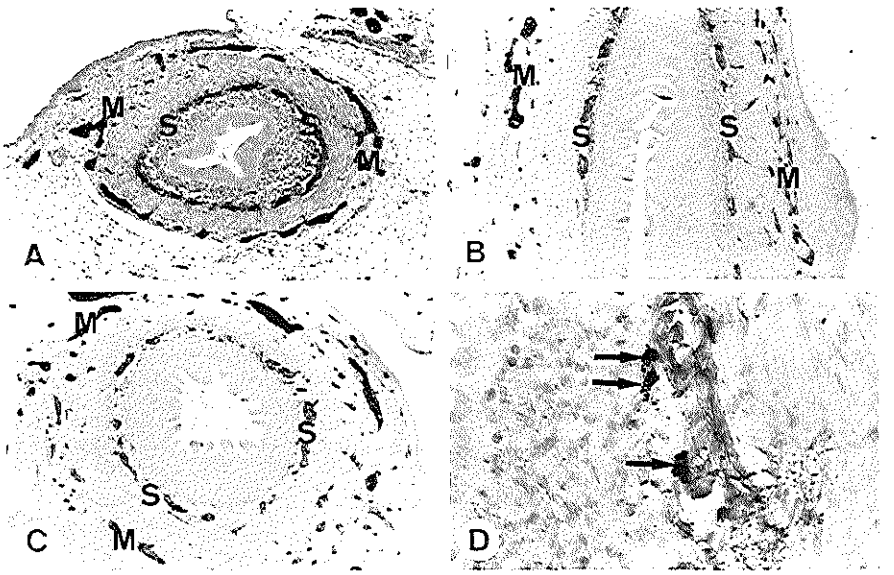

Fig. 5. Paraffin sections of cocultures of parts of quail E2 neural anlage and E4 chicken hindgut stained with the HNK-1 antibody to show the presence of myenteric (M) and submucous (S) ganglia. A: Neural anlage from the level of somites 1–3. B: Neural anlage from the level of somites 4–7. C: Neural anlage from the level of somites 6–7. ×25. D: Detail of Figure 5C showing melanocytes (arrows). ×63.

the gut (Smith et al., 1977; Newgreen et al., 1980; Peters-van der Sanden et al., 1993), whereas vagal neural crest cells rarely gave rise to melanocytes. Our results could indicate that the neural crest of somites 6–7 should be considered trunk neural crest. It is interesting to note that the caudal boundary of rhombomere 8 of the hindbrain is thought to lie between somites 5 and 6. An additional argument that the neural crest of somites 6 and 7 should be considered trunk neural crest comes from the observation that this is the most anterior level at which dorsal root ganglia, which are specific trunk derivatives, are formed (Lim et al., 1987).

Temporal Specification Within the Vagal Neural Crest With Regard to the Formation of Enteric Ganglia

We found that vagal neural anlagen taken from embryos having 20 or more somites, were still capable of giving rise to enteric neurons. Studies using quail-chick chimeras showed that ENS precursors leave the vagal neural anlage prior to the 13 somite stage, although migration sometimes lasts until after the 16 somite stage (Le Douarin and Teillet, 1973). Our results could indicate that the precursors for enteric ganglia either emigrate later than hitherto assumed, or remain in close contact with the neural tube for a prolonged time-period. In our study, vagal neural anlagen were dissected without the use of digestive enzymes, thereby possibly including neural crest cells which had already emigrated from the neural tube, but still remained in close contact. It is somewhat puzzling that neural crest taken from younger embryos, still containing all neural crest cells, gave rise to a less than normal amount of enteric ganglia. This could be related to a phenomenon described by Kirby (1989), who found that the addition of mesencephalic neural crest at the level of the cardiac crest, interfered with the development of the endogenous cardiac neural crest. It could also be that the commitment of the neural crest cells during the prolonged contact with the neural tube, depends on an in vivo process that can not fully occur in a coculture system. Smith et al. (1977) and Newgreen et al. (1989), using the same coculture system, described normal innervation of the hindgut after coculture with the vagal neural crest from stage 10 embryos. From their data, however, we could not determine whether the size and the amount of enteric ganglia they found resembled our cocultures with neural crest from younger or older embryos.

The first clues to the molecular mechanisms underlying specification within the vagal neural crest regarding ectomesenchymal and ganglionic derivatives,

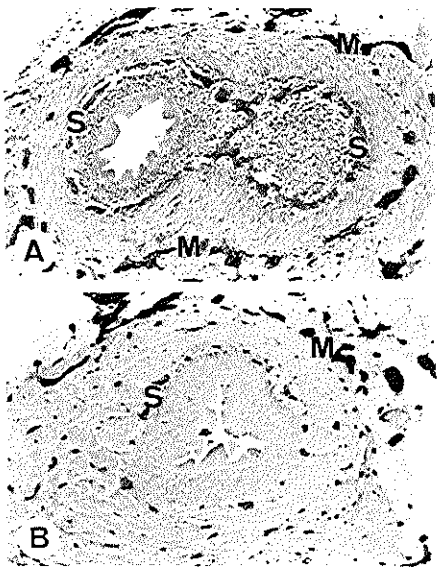

Fig. 6. Paraffin sections of cocultures of quail vagal neural anlage and E4 chicken hindgut. A: Vagal neural crest obtained from an embryo with 28 somites: HNK-1 immunoperoxidase staining shows the presence of a normal amount of myenteric (M) and submucous (S) ganglia. ×25. B: Vagal neural crest obtained from an embryo with 18 somites: enteric ganglia are present, but they are smaller and less abundant than in Figure 4A. ×25.

comes from studies with transgenic mice. A knock-out mutation of the *hox-1.5* gene (Chisaka and Capecchi, 1991) results in a phenotype that somewhat resembles the human DiGeorge anomaly, characterized by partial or total absence of the thymus and parathyroids often combined with cardiac outflow tract anomalies. All these defective organs receive an ectomesenchymal contribution from the vagal neural crest. A knock-out mutation of the *hox-1.6* gene (Lufkin et al., 1991; Chisaka et al., 1991) specifically affects the neurogenic crest of the hindbrain, whereas overexpression of the *hox-1.4* gene results in ENS defects (Wolgemuth et al., 1989; Gershon and Tennyson, 1991). Therefore, within the same region of the hindbrain (rhombomeres 4–7), populations of neural crest cells (neurogenic and ectomesenchymal) may be differentially specified by several *hox* genes that belong to the same cluster, but that exhibit different spatial and temporal patterns of expression.

EXPERIMENTAL PROCEDURES

Animals

Fertilized chicken—*Gallus gallus domesticus*—and quail—*Coturnix coturnix japonica*—eggs were incubated at 38°C in a forced draught humidified incubator. Chicken embryos were staged according to the table of Hamburger and Hamilton (1951), quail embryos were staged according to their number of somites. We used quail neural primordia and chicken hindgut in our coculture experiments. The quail condensed heterochromatin was used as a marker to detect the presence of quail neural crest cells in the chicken hindgut (Le Douarin and Teillet, 1973).

TABLE 3. Colonization Assay With Vagal Neural Crest From Embryos of Different Ages[a]

		Enteric ganglia		
Age (S)	n	+	±	−
9–18	10	1	5	4
20–28	10	9	0	1

[a]Age of the embryos is indicated by the total number of somites (S). The amount of enteric ganglia in the cocultures is scored: +) normal amount of ganglia as compared with in vivo, ±) fewer ganglia present than in vivo, −) no enteric ganglia present. n is the total number of cocultures in each group.

Neural Crest Ablation

Embryos were incubated for approximately 30 hr until they reached stages 8–10. Experimental animals were stained in situ with 0.02% neutral red through a window in the shell prior to carefully tearing the vitelline membrane in order to expose the neural folds. Portions of neural folds within the vagal neural crest region were ablated bilaterally by microcautery as has been described previously (Kirby et al., 1983). Shams were windowed and stained, and the vitelline membrane was torn, but the embryos were not altered. The windows were sealed and incubation was continued. The embryos were harvested after eleven days of incubation and fixed in either 4% paraformaldehyde or 70% ethanol containing 150 mM NaCl. After fixation the embryos were dissected and the isolated gut was divided into proximal and distal parts.

Colonization Assay

A 1 mm piece of hindgut just distal to the cecal bulges was isolated from 4-day-old chicken embryos (stage 22/23). At this developmental stage, this part of hindgut does not contain neural crest cells, neither vagal nor sacral (Pomeranz et al., 1991; Luider et al., 1992). The vagal neural crest adjacent to the first seven somites was isolated from quail embryos having 9–28 somites (stages 10–16). Neural tubes containing the neural crest were dissected and freed of somites using a microscalpel. No digestive enzymes were used. The neural tubes were divided in small parts with a length equivalent to 2 or 3 somites. Each part was placed on an Immobilon P millipore filter (Schleicher and Schuell, FRG) together with a segment of hindgut. The filter was placed upside down on the chorioallantoic membrane of a 7-day-old chicken host embryo. Trans-

plants were harvested after a 7-day coculture period and fixed in 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 24 hr at room temperature.

Immunohistochemistry

Specimens were routinely prepared for paraffin embedding and sectioned at 5 μm. We used the monoclonal antibodies HNK-1 (Abo and Balch, 1981) (ATCC; TIB 200; hybridoma supernatant, undiluted), as a marker for neural crest cells and enteric ganglia, and RMO 270 (Lee et al., 1987) (hybridoma supernatant, diluted 1:500) as a marker for neurofilaments. These first step antibodies were incubated for one hour at room temperature. Rabbit-anti-mouse immunoglobulins coupled to horseradish peroxidase (diluted 1:100; Dakopatts, Denmark) and goat-anti-mouse immunoglobulins coupled to FITC (diluted 1:20; Dakopatts, Denmark) were used as second step antibodies. PBS containing 0.1% Tween 20 was used for all rinsing. The peroxidase was visualized with 0.1% 3,3′ diaminobenzidine·HCl (Serva, FRG) with 0.01% H_2O_2. Endogenous peroxidases were inhibited by a 20 min incubation in methanol/hydrogen peroxide (99:1 v/v) solution. Immunoperoxidase stained sections were counterstained with haematoxylin for 1 min. To visualize the quail condensed heterochromatin marker, sections were incubated with Hoechst 33258 (2 μg/ml PBS) for 4 min. The sections were analyzed using a Leitz orthoplan fluorescence microscope. Immunoperoxidase stained sections were analyzed using a Leitz orthoplan microscope and photographs were taken with a Leitz camera using Agfa ortho film (25 ISO) and a Kodak Wratten 49B filter.

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CHAPTER 3.3.

Differential effect of retinoic acid on ectomesenchymal and ganglionic derivatives of the posterior rhombencephalic neural crest in chicken embryos

Marjo J.H. Peters-van der Sanden, Marie-Josée Vaessen, and Carel Meijers.

Abstract

Embryonic exposure to 13-cis or all-trans retinoic acid (RA) results in a spectrum of congenital malformations in humans and in a number of animal species. These malformations include central nervous system (CNS), craniofacial, cardiovascular, thymic and limb defects. A common denominator for a great majority of these malformations is the rhombencephalic neural crest. The affected outflow tract of the heart and the thymus receive a contribution from the vagal neural crest, overlying rhombomeres 5-8, which also gives rise to the neurons and supportive cells of the enteric nervous system (ENS). In the developing ENS, two cellular RA binding proteins (CRABP-I and II) and a nuclear receptor for RA (RXR) are present, which could point to a role for RA in ENS formation. There have been, however, no reports neither in humans nor in animal studies of an adverse effect of RA on the development of the ENS. We studied the effect of RA on ENS development in chicken embryos and found that injection of 100µl of $2.5x10^{-5}$ M RA into the yolk-sac, at different time points in development, did not result in ENS defects, although it did give rise to CNS and cardiovascular malformations. Using a coculture system, in which vagal neural crest cells and aneural hindgut were cultured on the chorioallantoic membrane of a seven day old host embryo, we were able to test higher RA concentrations, which would be lethal in early development. We found that injection of 100µl of $5x10^{-4}$ M RA did still not result in ENS malformations. We conclude that a concentration of RA, which has a teratogenic effect on the ectomesenchymal component of the vagal neural crest, does not result in ENS malformations. After addition of $5x10^{-5}$ M RA to in vitro cultured vagal neural crest cells, many neural crest cells detached from the culture substrate, while others were unaffected. This could also suggest that there are subpopulations within the vagal neural crest which differ in their sensitivity to RA.

Introduction

Retinoic acid (RA) plays an important role in pattern formation in vertebrate embryos. In particular the limb and the central nervous system seem to employ RA during crucial phases of their development. In the developing chick limb bud, endogenous RA is differentially distributed along the anteroposterior axis (Thaller and Eichele, '87), and administration of additional RA by implanting RA-soaked beads, induces mirror-image duplications of the digits (Tickle et al., '82; Tickle, '91). Recent studies in chicken, mouse, *Xenopus* and zebrafish embryos on the effect of RA on the CNS, showed that a relatively low dose of RA given at mid-gastrula stages resulted in a defect in the anterior hindbrain and its associated structures (Holder and Hill, '91; Morris-Kay et al., '91; Papalopulu et al., '91; Sundin and Eichele, '92). A reduction of forebrain and anterior midbrain seems to be specific for *Xenopus* embryos (Durston et al., '89; Ruiz i Altaba and Jessel, '91).

Teratogenicity of 13-cis retinoic acid (isotretinoin) in humans has been described by Lammer ('85), who found that exposure to RA during the first trimester of pregnancy results in a spectrum of congenital malformations, including cleft palate, ear defects and mandibular underdevelopment, as well as central nervous system (CNS), heart, thymus and limb defects. The teratogenicity of 13-cis and of its isoform all-trans retinoic acid has been confirmed in several animal species, including mouse, rat, hamster and monkey (Shenefelt, '72; Fantel et al., '77; Kamm, '82; Webster et al., '86).

With the exception of the CNS and limb defects, the tissues affected by RA receive part of their mesenchymal component from the cranial neural crest (Noden, '78; Kirby et al., '83; Bockman and Kirby, '84), suggesting that neural crest cells could be the target involved in RA-induced malformations. The craniofacial mesenchyme originates from the neural crest of the mesencephalon and anterior rhombencephalon (rhombomeres 1-5) (Noden, '78). The outflow tract of the heart and the thymus receive a contribution from the neural crest of the posterior rhombencephalon (rhombomeres 6-8) (Kirby et al., '83; Bockman and Kirby, '84). The posterior rhombencephalic crest, which is also called the vagal neural crest, also gives rise to ganglionic derivatives, such as the neurons and supportive cells of the enteric nervous system (ENS), the parasympathetic ganglia of the heart (cardiac ganglia), and dorsal root ganglia. The ENS constitutes the intrinsic nervous system of the digestive tract consisting of submucous and myenteric plexuses located on either side of the smooth muscle layer (Yntema and Hammond, '54; LeDouarin and Teillet, '73; Allan and Newgreen, '80). In chicken embryos, the formation of the ENS takes place between day two (E2), when neural crest cell migration starts, and day eight (E8), when ganglion formation in the hindgut is completed (Meijers et al., '87). Although there is evidence of a teratogenic effect of RA on the ectomesenchymal derivatives of the vagal neural crest, there are no reports of an adverse effect of RA on the development of the ENS. Clinicians, however, are frequently confronted

with the question, if exposure to RA may have been involved in ENS malformations, such as in isolated or syndromic cases of Hirschsprung's disease.

At the cellular level, RA enters the cell and, after reaching the nucleus, forms a complex with specific receptors. This RA-receptor complex is subsequently able to regulate the expression of a number of genes. The two cellular RA binding proteins (CRABP-I and CRABP-II), present in the cytoplasm, could either function as a shuttle to transport RA to the nucleus, or they could regulate the concentration of free cytoplasmatic RA either through steepening of an RA gradient or through functioning as a sink protecting the nucleus from excess RA. The latter possibility is favoured by a study in F9 teratocarcinoma cells in which CRABP-I was shown to influence the metabolism of intracellular RA (Boylan and Gudas, '92). Additional evidence for a protective role of CRABP-I against excess RA comes from the fact that CRABP-I has been found to be specifically expressed in tissues that appear to be sensitive to RA exposure during development, such as the posterior rhombencephalic crest (Vaessen et al., '90; Maden et al., '91; Ruberte et al., '91). In the gut of chicken embryos, the presence of both an RA receptor (RXR) (Rowe et al., '91) and CRABP-I and CRABP-II (Maden et al., '89; Ruberte et al., '92) has been described. The RXR receptor and CRABP-I have been found in enteric ganglia. CRABP-I has also been found in neural crest cells present in the wall of the gut prior to ganglion formation (Maden et al., '89). CRABP-II is only expressed in the epithelium of the gut (Ruberte et al., '92). The presence of both an RXR receptor and CRABP in the gut suggests that RA might play a role in ENS development.

A recent study in rat embryos (Kuratani and Bockman, '92) showed that administration of bisdiamide, an agent which gives rise to malformations similar to RA (Taleporos et al., '78), resulted in disturbed vagal nerve branching to the gut. Furthermore, it has been demonstrated in hamster embryos, that almost every organ or tissue can be affected by RA, if the embryo is treated with a specific dose and at the critical period of development (Shenefelt, '72). Therefore, we studied whether ENS malformations could be induced by RA in chicken embryos, using two experimental approaches. First, we performed in vivo experiments in which we administered a teratogenic, sublethal dose of RA at different time-points in development. Furthermore, we used a coculture system, in which we cultured the vagal neural crest and a piece of aneuronal hindgut on the chorioallantoic membrane of a seven day host embryo. With this coculture system, we tested the effect of a ten-fold higher dose of RA, which would be lethal in vivo.

Materials and methods

Animals

Fertilized chicken -*Gallus gallus domesticus*- and quail -*Coturnix coturnix japonica*- eggs were incubated at 38°C in a forced draft humidified incubator. Chicken embryos were staged according to Hamburger and Hamilton

('51), quail embryos were staged according to the number of somites.

In vivo administration of RA

A stock solution of 10^{-2} M all-trans retinoic acid (Sigma, USA) was made in DMSO (dimethyl-sulfoxide; Merck, Germany). This stock solution was diluted in phosphate buffered saline (PBS) to a working concentration of $2.5x10^{-5}$ M. 100µl of this solution was injected into the yolk-sac of a chicken egg through a punctured hole in the shell at two different time points in development: day 0 (E0 or blastoderm stage) and 4 (E4) of incubation. The hole in the shell was closed with scotch tape. Controls were injected with 100µl of 0.25% DMSO in PBS or with PBS alone. Stage 15 embryos were visualized through a window in the egg-shell and treated with 1.0 µg RA in 1µl 2% DMSO, which was applied onto the vitelline membrane using a Hamilton syringe. After a total of 9 or 10 days of incubation, when neural crest cell colonization is completed, embryos were harvested and examined macroscopically. The gut was isolated and fixated in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned at 7 µm and prepared for immunohistochemistry.

RA treatment of cocultures of E2 quail vagal neural anlage and E4 chicken hindgut

Chorioallantoic membrane cocultures were performed as described previously (Peters-van der Sanden et al., '93). Briefly, a 1 mm segment of hindgut just distal to the caecal bulges was isolated from four-day-old chicken embryos (E4; HH stage 22/23). At this developmental stage, this part of the hindgut does not contain neural crest cells (Teillet et al., '78; Pomeranz et al., '91; Luider et al., '92). Vagal neural anlagen, including neural crest, neural tube and notochord, were isolated from stage 10-12 quail embryos. The excised neural anlage was placed on an Immobilon P filter (Schleicher and Schuell, FRG) together with the segment of hindgut. This filter was then placed upside down on the chorioallantoic membrane of a seven-day-old chicken embryo and cultured for seven days. 100µl $5x10^{-4}$ M RA was injected into the yolk-sac of the seven-day-old host embryo immediately at the start of the coculture or after an initial three day coculture period, when neural crest cells first start to colonize the gut. Controls were injected with 100µl 5% DMSO.

In order to test whether the administered RA reached the site of the graft, embryonic stem cells carrying a construct with part of the RARß promoter containing an RA response element in front of the lacZ reporter gene (kind gift of Dr. P. van der Saag), were grafted on the chorioallantoic membrane. After 48 hours the chorioallantoic membrane containing the grafted cells was harvested and fixated in 2% paraformaldehyde/0.2% glutaraldehyde in PBS and stained for ß-galactosidase activity (Sanes et al., '86).

Chorioallantoic membrane cocultures were also performed with E2 chicken vagal neural primordia isolated from embryos treated at E0 with $2.5x10^{-5}$ M RA according to the in vivo protocol (see above). After a total coculture period of seven days, transplants were harvested and fixated in 4% paraformaldehyde in PBS. Transplants were embedded in paraffin, sectioned at 7 µm and prepared for immunohistochemistry.

RA treatment of in vitro cultured vagal neural crest cells

Vagal neural primordia from two-day-old quail embryos were dissected and cultured as described previously (Peters-van der Sanden et al., '93). RA was added immediately at the start of the culture in a final concentration of $5x10^{-5}$ or $5x10^{-6}$ M in 0.5% DMSO. Culture medium was changed after two days of culture and a second dose of RA was administered. Cultures were examined morphologically every day with a phase-contrast microscope. After a four-day culture period cultures were washed, fixated in 2% paraformaldehyde in PBS for 30 min at room temperature, and stained with HNK-1 (see immunohistochemistry).

Immunohistochemistry

We used the monoclonal antibody HNK-1, a marker for neural crest cells and enteric neurons, as primary antibody (Abo and Balch, '81) (ATCC; TIB 200; hybridoma supernatant, undiluted), incubated for one hour at room temperature. Rabbit-anti-mouse immunoglobulins coupled to horse-radish peroxidase (diluted 1:100; Dakopatts, Denmark) were used as a second step antibody. PBS containing 0.1% Tween-20 was used for all rinsing. Peroxidase was visualized with 0.1% 3,3'diaminobenzidine.HCl (Serva, FRG) / 0.01% H_2O_2 in PBS. Endogenous peroxidases were inhibited by a 20 min incubation in methanol/hydrogen peroxide (99:1 v/v) solution. Sections were counterstained with haematoxylin for one min. For immunoperoxidase, sections were analyzed using a Leitz orthoplan microscope and photographs were taken using Agfa ortho film (25 ISO) and a Kodak Wratten 49B filter.

Results

Effect of RA administration at various developmental stages in vivo

Of the 20 eggs injected with $2.5x10^{-5}$ M RA at E0, corresponding to the blastoderm stage, and examined at E2, 15 contained live embryos. Eight embryos did not show macroscopically visible malformations (Fig. 1A), although four showed signs of growth retardation. Of the

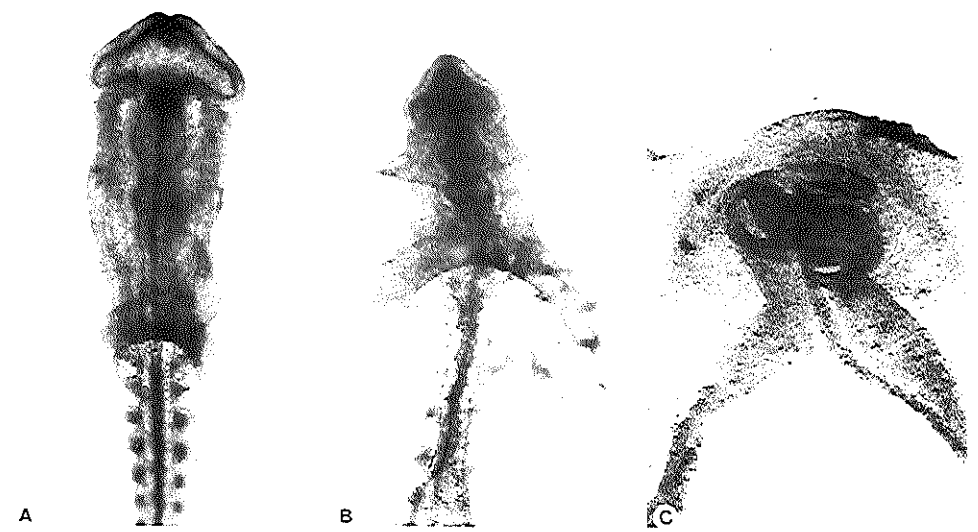

Figure 1: *Micrographs of embryos after injection of $2.5x10^{-5}$ M RA at E0 (blastodermstage) and examined macroscopically at E2. A) Stage 11 embryos with no macroscopically visible malformations. B) Embryo of comparable age as in A; both the cranial and trunk region are smaller than in control embryos and clearly malformed. C) Embryo, whose development arrested during gastrulation and which is clearly malformed.*

remaining seven embryos, three had arrested development during gastrulation (Fig. 1B), while four had passed through gastrulation, but showed CNS malformations in the hindbrain region and/or disturbances in somitic segmentation in the trunk region (Fig. 1C). Of the 15 eggs injected with 0.25% DMSO at E0 and examined at E2, 11 embryos were recovered, seven of which were normal and four of which showed growth retardation. None of these embryos, however, were malformed. Of the 10 eggs treated with PBS at E0 and examined at E2, seven embryos were recovered, which were of normal size and showed no malformations. Of the 20 eggs injected with RA at E0 and examined at E9, only three live embryos were recovered. The other embryos had started development, but had died prior to stage 24. The three embryos that had survived did not show any macroscopical malformations. The gut of these embryos showed a normal morphology with clear presence of myenteric and submucous ganglia on either side of the circular smooth muscle layer (Fig. 2A). The ganglia were visualized with the HNK-1 antibody, a marker for early migrating neural crest cells and their neuronal derivatives (Vincent et al., '83; Vincent and Thiery, '84). Of the 15 eggs injected with DMSO at E0 and examined at E9, seven live embryos were recovered. These looked normal macroscopically and the gut of these embryos contained a normal ENS. The five eggs injected with PBS and examined at E9 gave rise to one live embryo which was normal macroscopically and had a normal ENS. Table 1 summarizes the results obtained at E2 and E9.

We also examined the ENS of E9 embryos treated with 1 μg RA at E2 (stage 10-15), the time when neural crest cells are migrating from the posterior rhombencephalic crest on their way to their target organs. In an earlier study, these embryos were shown to have malformed outflow tracts of the heart characterized as double outlet right ventricle (DORV) (Broekhuizen et al., '92). The gut of these embryos, however, showed no abnormalities and a normal pattern of enteric ganglia was observed (Fig. 2B) (n=3). DMSO-treated control embryos from this study, which showed no macroscopical abnormalities in the outflow tract of the heart, also contained a normal ENS.

Injection of 2.5×10^{-5} M RA into the yolk-sac of embryos at E4, when neural crest cells have all migrated from their site of origin and for the most part have already reached their target organ, had no macroscopically detectable effects on survival and subsequent embryonic development (examined at E10). The ENS (Fig. 2C) had developed normally (n=4). These results show that in vivo administration of RA at various time-points in development and in a concentration that clearly affects certain aspects of embryonic development, had no effect on ENS formation.

Table 1: Survival of embryos injected with 100 µl $5x10^{-5}$ M RA prior to incubation

Treatment	Examined at E2		Examined at E9	
RA	n=20	5 died 7 malformed 4 growth retarded 4 normal	n=20	17 died 3 normal
DMSO	n=15	4 died 4 growth retarded 7 normal	n=15	8 died 7 normal
PBS	n=10	3 died 7 normal	n=5	4 died 1 normal

At E2 the surviving embryos were examined macroscopically for morphological defects. Observed malformations mainly entailed CNS defects in the hindbrain region. At E9 we studied both morphology and the innervation of the gut.

Effect of RA on ENS formation in chorioallantoic membrane cocultures

To test higher doses of RA, we performed chorioallantoic membrane cocultures of vagal neural anlage isolated from normal E2 quail embryos, and E4 chicken hindgut. The E7 host embryos were injected with 100µl $5x10^{-4}$ M RA or 0.5% DMSO into the yolk-sac. This high dose of RA, which is lethal during early embryonic development, had no detectable adverse effect on the seven-day-old host embryo. Injection of RA was performed either at the start of the coculture (n=3), or after an initial coculture period of 3 days (n=3). The 3 day culture period was chosen, because at this time neural crest cells are first observed in the hindgut in the coculture system (unpublished results). The cocultures all contained a normal amount of enteric ganglia (Fig. 3), as did the DMSO controls. In the cocultures injected with RA after 3 days, however, the shape of the enteric ganglia differed somewhat from controls, the ganglia being round instead of elongated.

In order to test whether the administered RA reached the graft site, we grafted embryonic stem cells, carrying a RA response element/lacZ reporter transgene, to the chorioallantoic membrane and cultured these for 2 days. Cells on the chorioallantoic membrane of an egg injected with $2.5x10^{-4}$ M RA were intensely blue after a 5 hour staining period, indicating lacZ gene activity. The cells were predominantly located adjacent to blood

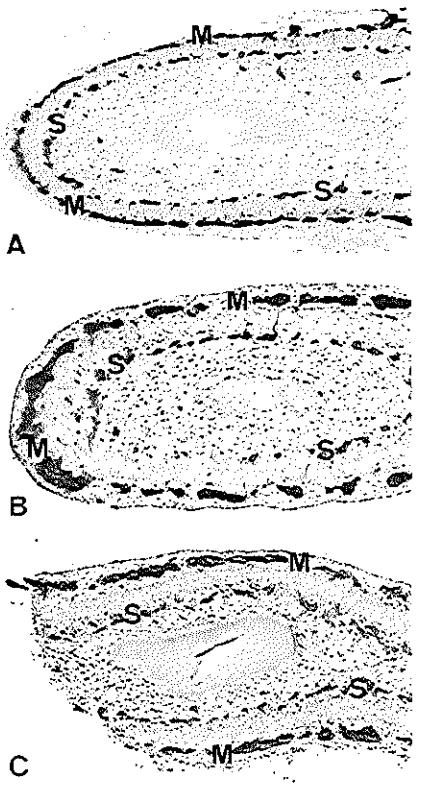

Figure 2: Paraffin sections of the gut of E9 chicken embryos after treatment with $2.5x10^{-5}$ M RA at different time-points in development, stained with the HNK-1 antibody. A) Injection of RA into the yolk-sac at E0. B) Direct application of RA onto the vitellin membrane at stage 15. C) Injection of RA into the yolk-sac at E4. In all three cases, the gut contains a normal ENS with myenteric (M) and submucous (S) ganglia. 16x

vessels. Cells grafted to control eggs, injected with DMSO, stained lightly blue after the same staining period. This could be caused by the endogenous RA concentration, because in vitro experiments showed that the cells are already sensitive to 10^{-8} M RA, which is the physiological RA concentration in chicken eggs. The blood vessels of control chorioallantoic membranes not containing cells did not stain (Fig. 4).

Vagal neural primordia, isolated from the E2 chicken embryos treated with RA, DMSO or PBS at E0 according to the in vivo protocol, were used in chorioallantoic membrane cocultures. All vagal neural primordia, including those derived from affected embryos, were capable of giving rise to a normal ENS in the coculture system (Fig. 3C).

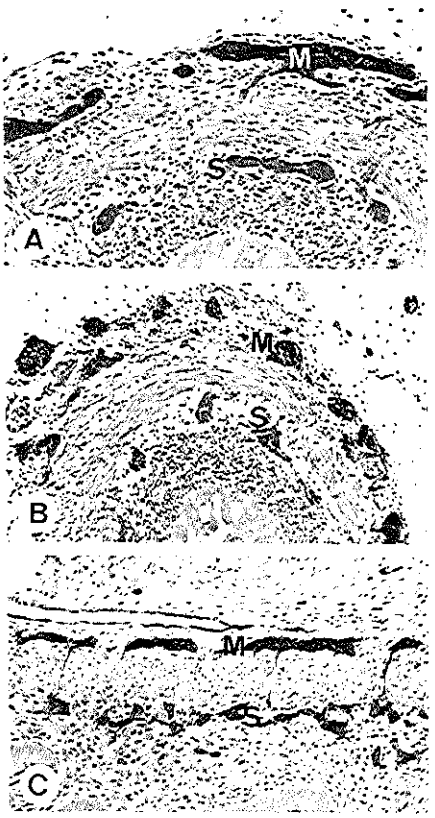

Figure 3: Paraffin sections of 7-day cocultures of E2 vagal neural anlage and E4 chicken hindgut, stained with the HNK-1 antibody. A) E7 host embryo injected with $2.5x10^{-4}$ M RA at the start of the coculture: the hindgut contains a normal ENS with both myenteric (M) and submucous (S) ganglia. 25x B) E7 host embryo injected with $2.5x10^{-4}$ M RA after 3 days of coculture: myenteric (M) and submucous (S) are present, but are of somewhat abnormal shape being round instead of elongated. 25x C) Coculture with E2 vagal neural anlage isolated from embryos injected with $2.5x10^{-5}$ M RA at E0: the hindgut contains a normal amount of myenteric (M) and submucous (S) ganglia, which are of normal shape. 25x

RA affects cell-substratum adhesion of vagal neural crest cells cultured in vitro

After one day of in vitro culture, RA treated quail neural crest cell cultures did not differ from control cultures treated with DMSO. The cultures consisted of about one to two thousand small stellate cells, which had a flattened morphology indicative of firm attachment to the culture substratum. After two days of culture, cells became rounded and part of the cells detached from the surface and formed small aggregates. This effect was most clearly observed in cultures treated with $5x10^{-5}$ M RA, but could also be observed in cultures treated with $5x10^{-6}$ M or to a lesser extent with DMSO alone. After three days of culture even more cells had detached, but even after five days part of the cells remained firmly attached to the surface. Figure 5 shows RA treated cultures fixated after 4 days and stained with the HNK-1 antibody. Both in the RA treated cultures and in the DMSO controls, the percentage of HNK-1 positive cells is low, which is normal for vagal neural crest cells cultured for 4 days (Peters-

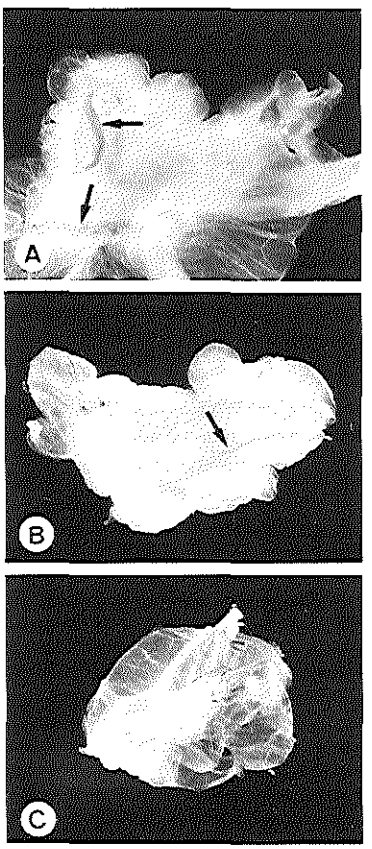

Figure 4: *Micrographs of chorioallantoic membranes of E7 chicken embryos grafted with murine ES cells carrying a RA response element/LacZ reporter transgene, stained for ß-galactosidase activity after 2 days of culture. A) Chorioallantoic membrane containing ES cells exposed to $2.5x10^{-4}$ M RA: cells are mainly localized along blood vessels, where intense staining is visible (arrows). B) Chorioallantoic membrane containing ES cells not exposed to exogenous RA: cells, again localized along blood vessels, are lightly stained (arrow). C) Chorioallantoic membrane without ES cells and without exposure to exogenous RA: there is no staining along blood vessels, but there is some aspecific staining present.*

van der Sanden et al., '93). The cells show a variable morphology ranging from flat stellate to bipolar. After replating in medium without RA, treated cells reattached to the surface and looked normal, which suggests that detachment of cell was not caused by cytotoxicity of RA.

Discussion

In this study, we examined the effects of RA on ectomesenchymal and ganglionic derivatives

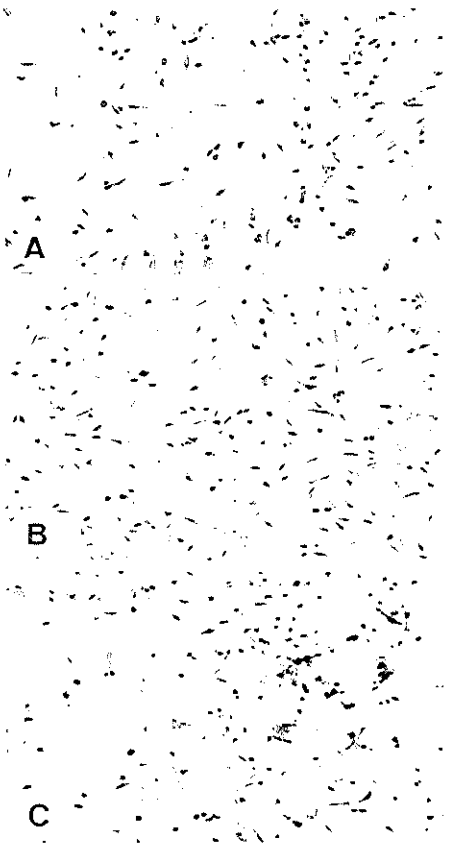

***Figure** 5: Micrographs of quail vagal neural crest cells cultured for 4 days in vitro and stained with the HNK-1 antibody. A) Neural crest cells that remained attached to the culture substrate in the presence of $2.5x10^{-5}$ M RA. B) Neural crest cells cultured in the presence of $2.5x10^{-6}$ M RA. C) Neural crest cells cultured in the presence of 0.5% DMSO. In all three cases the morphology of the cells is similar varying from flat, stellate to round or bipolar. The percentage of HNK-1 positive cells was very low. 16x*

of the posterior rhombencephalic (vagal) neural crest. We compared the effect of RA on the ENS, a ganglionic derivative of the vagal neural crest, with the effect on ectomesenchymal derivatives of the vagal neural crest, particularly the cardiac outflow tract, known to be affected by RA exposure. We found that injection of RA into the yolk-sac at the blastoderm stage (E0) had varying effects, ranging from disturbed gastrulation, defects in the hindbrain region, growth retardation or embryonic death. The embryos that survived until E9 were all normal macroscopically and contained a normal ENS. This could be due to a selection process in which only those embryos that were not, or only slightly affected by the RA treatment survived. Therefore, we performed chorioallantoic membrane cocultures of the neural anlage isolated from E2 embryos, that were clearly affected by RA treatment at E0, and E4 hindgut, and found that vagal neural crest cells from RA affected embryos were still capable of giving

rise to a normal ENS. We also examined the ENS of embryos, which were treated with RA through direct administration onto the vitelline membrane at stage 15 of development (E2). This treatment was shown to result in cardiovascular defects in the outflow tract of the heart, characterized as double outlet right ventricle (DORV) (Broekhuizen et al., '92), but had no effect on ENS formation. These results show that in vivo treatment with RA in a dose which clearly affects several aspects of embryonic development including the development of ectomesenchymal derivatives of the posterior rhombencephalic neural crest, has no adverse effect on ENS formation.

There are two main differences between the two in vivo treated experimental groups, which could have influenced the effects of RA. The first difference is the time-point at which RA was administered, E0 versus E2 (stage 15). RA given at E2 can not affect gastrulation and CNS development which occur prior to that stage. The second difference is the way RA is administered to the embryo. In the embryos treated at E0, 100µl of $2.5x10^{-5}$ M RA ($=2.5x10^{-3}$ µmol) was injected into the yolk-sac, whereas in embryos treated at E2, 1 µg of RA ($=3x10^{-3}$ µmol) was applied directly onto the vitelline membrane. This might influence the effective RA concentration that reaches the embryo. Injection of 100µl $2.5x10^{-5}$ M RA would result in an overall RA concentration of $2.5x10^{-8}$ M, if RA is distributed evenly over the yolk-sac (estimated egg volume: 100ml). Administration of 1 µg of RA would result in an overall RA concentration of $3x10^{-8}$ M, but because the RA is applied directly onto the vitellin membrane, the effective dose reaching the embryo could be much higher. One way to circumvent this problem is to culture early chicken embryos in vitro and adding RA to the culture medium and thus controlling the RA concentration reaching the embryo (Sundin and Eichele, '92).

Injection of $2.5x10^{-3}$ µmol RA into the yolk-sac of E4 embryos did not result in malformations at E9 in our study, neither in the cardiovascular system nor in the ENS. Cardiovascular defects (type I ventricular septum defects), however, were found after administration of higher doses of RA to E4 embryos (Jelinek and Kistler, '81; Hart et al., '90), but in these studies the development of the ENS was not investigated. Administration of this high dose of RA to E2 embryos resulted in a percentage of embryonic death of nearly 100% (Jelinek and Kistler, '81; Hart et al., '90). In our study, we also observed a considerable amount of embryonic death. Combining the results of embryos examined at E2 and E9 showed, however, that embryonic death did not differ significantly between the RA (55%) and DMSO (46%) treated groups. The fact that even control injections with PBS resulted in a high percentage of embryonic death (46%), could indicate that the injection procedure alone is deleterious for the embryos. It has been shown that injection of a certain amount of any fluid into the yolk-sac can be deleterious for embryonic survival (Wyatt and Howarth, '76). 100 µl appeared to be to maximum amount of fluid, that can be tolerated for embryonic survival.

In our study, PBS injection never resulted in abnormalities in surviving embryos,

whereas injection with either DMSO or RA resulted in growth retardation. Only RA injection, however, resulted in visible malformations in embryos examined at E2, consisting mainly of CNS disturbances in the hindbrain region. The fact that no malformed embryos were found at E9 could be due to lethality of the malformations between E2 and E9. Our results indicate that, apart from having a possible effect on embryonic survival, DMSO affects embryonic growth. There have been some reports on an adverse effect of DMSO on embryonic development (Wyatt and Howarth, '76; Hart et al., '90), but DMSO was never found to induce abnormalities in structures with a neural crest derivation or contribution.

In a second experimental approach, we tested the effect of RA in a coculture system. Injection of 100µl $2.5x10^{-4}$ M RA at the start of the 7 day coculture period did not affect ENS formation, whereas injection of the same amount of RA after an initial 3 day culture period resulted in the presence of a normal amount of enteric ganglia, which were, however, round instead of elongated. This might indicate that there is a difference in RA sensitivity between the early and late phases of ENS formation. Whereas migration of vagal neural crest cells to the gut and homing to the correct sites seem to be insensitive to RA, aggregation into a correct pattern of enteric ganglia might be influenced by RA. This could be related to the disturbed interaction between vagal neural crest cells and the extracellular matrix observed after RA exposure in vitro. The intense blue staining of the ES cells carrying an RA response element/lacZ reporter served as proof that the administered RA reached the chorioallantoic membrane.

RA treatment of in vitro cultured vagal neural crest cells gave similar results as RA treatment of mesencephalic and trunk neural crest cells (Thorogood et al., '82; Smith-Thomas et al., '87). These studies showed that both mesencephalic and trunk neural crest cells responded to RA with a change in morphology and detachment from the culture substrate. Vagal neural crest cells also responded to RA by detaching from the culture substrate and forming small aggregates. This suggests that RA causes a decrease in cell-substrate and an increase in cell-cell adhesion resulting in impaired cell-extracellular matrix interactions. This effect is probably not specific for neural crest cells and occurs in all migrating cells (Thorogood et al., '82). Although many neural crest cells detached from the surface, a significant amount of neural crest cells remained firmly attached to the surface. This latter population of cells could represent neural crest cells which are not sensitive to RA.

We conclude that, in chicken embryos, a teratogenic dose of $2.5x10^{-3}$ µmol RA, which induces anterior hindbrain and cardiac malformations, does not induce malformations of the ENS at the developmental time-points tested. Furthermore, a ten-fold higher dose of RA ($2.5x10^{-2}$ µmol), which is lethal in vivo, does not disturb neural crest cell colonization of the hindgut in a coculture system. The relative insensitivity to RA of the ganglionic derivatives of the vagal neural crest could add further evidence to the hypothesis that there are distinct subpopulations of cells within the vagal neural crest.

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Colonization Characteristics of Enteric Neural Crest Cells: Embryological Aspects of Hirschsprung's Disease

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• This study explores the development of the enteric nervous system in avian embryos. Particular emphasis was given to colonization characteristics of neural crest cells present in primitive enteric ganglia. By coculturing neuronal and aneuronal gut of quail and chicken embryos, we investigated if and when neural crest cells in primitive enteric ganglia could detach from these ganglia, migrate, and colonize adjacent chicken gut. Quail neural crest cells were identified using the quail nucleolar marker and the HNK-1 antibody. Enteric neurons were identified using three monoclonal antibodies directed against neurofilament proteins. We found that neural crest cells detached from primitive ganglia in neuronal quail gut from E6 till E9, whereas neural crest cells did not leave enteric ganglia from E10 gut. These observations show that there is a transient phase during which enteric neural crest cells can leave the gut. To determine whether neural crest cells could colonize neuronal gut we cocultured neuronal gut or the neural primordium and neuronal chicken gut (E11). We found that quail neural crest cells do not colonize neuronal E11 gut, whereas they do colonize aneuronal gut of the same age. We suggest that aneuronal gut attracts neural crest cells by diffusing factors.

INDEX WORDS: Neural crest; enteric nervous system; chick embryo; Hirschsprung's disease; quail-chick chimera.

THE NEURAL CREST is a transient structure in vertebrate embryogenesis that leaves its imprint on the entire embryo. Diseases arising from the neural crest are particularly diverse in clinical presentations, involving endocrinologic, cutaneous, neurological, digestive, pulmonary, or other types of syndromes[1-3] because neural crest cells invade almost every organ of the body.[4-6] Knowledge of the processes involved in neural crest cell adhesion, migration, and homing within the target organ is essential for understanding the pathogenesis of congenital malformations related to the neural crest.

Enteric neurons and their supportive cells together with extrinsic nerve fibres constitute the enteric nervous system (ENS). The neural crest origin of enteric neurons was demonstrated by a variety of experimental approaches.[7,8] "Vagal" neural crest cells, adjacent to somites 1 to 7, migrate laterally and fill the third and fourth visceral arches. At the level of the pharynx they disperse alongside the developing bowel through the splanchnic mesoderm.[9,10] Within the bowel, neural crest cells migrate to the hindgut in a craniocaudal sequence.[11] Active craniocaudal neural crest cell migration coincides with active cell proliferation.[12] The migration of neural crest cells from the vagal neural crest to the hindgut is the longest migratory distance known. Neural crest cell colonization of the gut correlates with HNK-1–positive mesenchymal cells in the submucosa.[13] Neural crest cell adhesion within the enteric ganglia is mediated by N-CAM, Ng-CAM (the neuron-glia cell adhesion molecule), and N-cadherin.[14-16]

In previous experiments, we found that at an advanced stage of development aneuronal bowel still provides migratory substrates required for active neural crest cell migration.[17] In the present study we determined whether such migratory substrates persist in neuronal bowel. In order to supply distal bowel segments with neural crest cells, newly formed neural crest cell aggregates in proximal bowel segments might well take on the characteristics of the neural crest itself in the process of cell adhesion and cell detachment. In other words, a postmigratory neural crest cell might become or provide a migratory neural crest cell. To substantiate this premise, we investigated whether aggregated neural crest cells in enteric ganglia of quail embryos were able to colonize aneuronal segments of the chick gut.

MATERIALS AND METHODS

Embryos

White leghorn chicken embryos (*Gallus gallus domesticus*) and Japanese quail embryos (*Coturnix coturnix Japonica*) were staged according to the table of Hamburger and Hamilton.[18] Alternatively, the stage of young embryos was determined by counting the number of paired somites, and the stage of older embryos was determined according to the number of incubational days. Eggs were incubated at 38°C in a forced draft incubator at a relative humidity of 80%.

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Dissection of Embryonic Gut and Grafting Experiments

Segments of embryonic gut (quail and/or chick) were grafted alone or in combination onto the chorioallantoic membrane of E7 chick embryos after abrasion of the superficial layer, at the bifurcation of two great vessels. The grafts were fixed in position with a piece of cellophane (Clingo wrap sterilized in 70% ethanol and dried in air). The egg was sealed with adhesive tape and incubated for 1 week.

To determine the earliest arrival of neural crest cells in various segments of quail gut, we dissected from E3.5 until E11 quail embryos and cultured these on the chorioallantoic membrane. At E4 the postumbilical bowel was divided into 3, at E5 into 4, at E6 and E7 we used the 5 distal segments.

To determine whether neural crest cells in newly formed enteric ganglia can detach and colonize neighbouring aneuronal segments, we cocultured neuronal quail gut with aneuronal chick gut. For the neuronal quail gut we took a 1-mm segment, distal to the origins of the ceca from E6 to E11. Chicken hindgut (the region between the cecal primordia and the cloaca) was dissected from embryos before its colonization by neural crest cells, ie, at E4 and E5.[13]

To determine whether neural crest cells can colonize neuronal gut, we cocultured neuronal quail gut and neuronal chick gut. Neuronal quail gut was also grafted in combination with the vagal neural primordium. Segments of the neural tube (somite 1 to 7) were dissected from 12 to 21 somite embryos (E2) using tungsten needles. All dissections were performed in Ham's F10 tissue culture medium.

Tissue Fixation

For preparing cryosections, grafts were embedded in Tissue Tek II O.C.T. Compound (Miles, Naperville, IL) and snap-frozen in liquid nitrogen cooled isopentane and cryostat sectioned (10 μm). Cryostat sections were mounted on microscope slides coated with chrome alum. Before immunocytochemical incubation, sections were fixed in acetone for 5 minutes, and then dried in air. For preparing paraffin sections grafts were fixed in 4% formaldehyde in phosphate-buffered saline (PBS), dehydrated, embedded in paraffin, and sectioned at 5 to 7 μm.

Quail neural crest cells in chick host gut were identified using the DNA staining according to Feulgen and Rossenbeck.[14] Quail and chick cells could be distinguished on light microscopy by their different nucleolar structures.

Immunocytochemistry

The HNK-1 IgM antibody can be used to identify avian neural crest cells.[20] The HNK-1 hybridoma cell line was purchased from the American Tissue Culture Collection TIB 200.[21] HNK-1 immunoperoxidase staining was performed on both paraffin and cryo sections using undiluted supernatant.

Three mouse monoclonal antibodies, raised against purified human neurofilament triplet proteins but cross-reacting to chicken neurofilaments, were used to identify enteric neurons. The 3G6 Ig-M antibody is specific for the 160 kD and 200 kD NF-protein,[22] the 2F11 antibody (Sanbio, Holland) for the 160 and 200 kD,[23] and C90 for the 200 kD. NAPA-73, a neurofilament associated protein of 73 kD, is present in early populations of neural crest cells.[24] Supernatant containing the E/C8 IgM antibody was used undiluted.

Rabbit-anti-mouse peroxidase conjugated immunoglobulins (Dako, Copenhagen, Denmark) were used in a dilution of 1:100. In order to reduce background staining, we added 2% chick serum to the conjugate. Peroxidase was visualized by 0.1% 3,3' diaminobenzidine 4HCl (Serva, Heidelberg, Germany) and 0.02% hydrogen peroxide. All rinsing and diluting was done in PBS (pH 7.4) with 0.1% Tween-20.

RESULTS

HNK-1–Positive Cells in Embryonic Quail Gut

We determined the moment of neural crest cell arrival in various segments of embryonal quail gut by studying HNK-1 immunoreactivity in longitudinal sections (E5 to E11). Table 1 summarizes the HNK-1 immunoperoxidase findings. At E5, dispersed HNK-1–positive neural crest cells were observed between the undifferentiated mesenchyme of the gizzard. HNK-1 immunoreactive cells were not observed in the midgut and hindgut. Distal to the cecum HNK-1 immunoreactivity was only located in Remak's ganglion. From E5 onward Remak's ganglion remained positive. At E6, HNK-1–positive neural crest cells were located as aggregates in the differentiating muscle layers of the gizzard. HNK-1–positive neural crest cells at the site of the myenteric plexus were observed down to the umbilicus. The neural crest cells were still not grouped in ganglionic structures. Down to the cecum few HNK-1–positive neural crest cells were seen. At E7, HNK-1 immunoreactivity visualized myenteric plexuses in the periumbilical gut; however, the submucous plexus did not show HNK-1 immunoreactivity. Between the umbilicus and the cecum few HNK-1–positive neural crest cells were found, but these were not grouped in clusters of neural crest cells at the sites of the enteric ganglia. The distal 2 mm of hindgut did not show HNK-1 immunoreactivity. At E8, a myenteric and submucous plexus was observed in the entire bowel.

Chorioallantoic Cultures of Embryonic Quail Gut of Various Developmental Stages

To determine whether neural crest cells were present in different segments of the gut, we cultured explants of various parts of the gut and screened for the presence of HNK-1– and neurofilament-positive enteric neurons. Table 2 summarizes the results of this culture experiment. A striking observation was that gut segments that did not contain HNK-1–positive neural crest cells at the time of explantation

Table 1. HNK-1–Positive Cells in Successive Segments of Embryonic Bowel

	Day of Development							
Bowel Segment	E4	E5	E6	E7	E8	E9	E10	E11
Gizzard	—	+	+	+	+	+	+	+
Ventriculus	—	+	+	+	+	+	+	+
Umbilicus	—	—	±	+	+	+	+	+
Ceca			—	±	+	+	+	+
Colorectum	—	—	—	—	+	+	+	+

Table 2. HNK-1–Positive Enteric Neurons in Cultured Segments of Embryonic Quail Gut

Bowel Segment	Day of Explantation			
	E4	E5	E6	E7
Colorectum	2/2	2/4	1/3	2/3
Colon			2/2	4/4
Ceca		7/7	2/2	3/3
Prececal			2/2	1/1
Umbilicus		5/5	1/1	1/1
Preumbilical		1/1	2/2	1/1

did contain HNK-1–positive enteric neurons after the culture period. The HNK-1 immunoreactivity in the cultures was confined to the enteric ganglia and not in the serosa or the chorioallantoic membrane. This indicates that neural crest cells do not have the tendency to leave the gut in the culture system.

Neuronal Gut Innervates Aneuronal Gut in Culture

To determine whether neural crest cells in neuronal gut can detach and colonize an aneuronal hindgut segment, we cocultured aneuronal chick gut (E4) with neuronal quail gut E6 to E11 on the chorioallantoic membrane. Table 3 shows that aneuronal chick gut (E4) contained quail cells after coculture with neuronal quail gut from E6-E8 embryos (11/11). The quail cells were localized in the enteric ganglia. HNK-1 immunocytochemistry on serial sections showed that the quail cells were HNK-1 positive. No quail neural crest cells were found in the chick gut when neuronal quail gut (E10) was cultured together with aneuronal chick gut (1/12). The chick gut did not show E/C8 or NF immunoreactivity.

Neural Crest Cells Do Not Colonize Neuronal Gut

To determine whether quail neural crest cells are able to colonize neuronal gut we cultured neuronal chick hindgut E11 together with neuronal quail gut (E6). Table 3 summarizes the results of both immunocytochemical analysis and the staining for the quail nucleolar marker. We never observed quail neural crest cells in the region of enteric ganglia (0/10). Special attention was paid to look for quail cells at remote sites. Quail neural crest cells were not observed close to the neuronal gut segment of the chicken embryo. Apparently, quail neural crest cell do not have the capacity to colonize embryonal neuronal chicken gut.

Table 3. Cocultures of Quail Neural Crest Cell Donors and Chick Recipient Gut

Donor Quail Neural Crest Cells in Neuronal Gut	Chick Recipient Gut		
	CE4	CE11	CE4 + 7CAM
E6	10/11	0/10	6/13
E7	11/11	ND	ND
E10	1/12	ND	ND
Neural primordium E1.5	8/8	1/10	7/11

Abbreviation: ND, not determined.

To exclude the possibility that the invasive potential of migrating neural crest cells in the bowel is less extensive compared to original quail neural crest cells, we cocultured neuronal chick gut (E11) with the vagal neural primordium of quail E1.5. Only 1 of 10 neuronal bowel segments contained some quail cells after coculture. In contrast, if the quail neural primordium of E1.5 or neuronal quail gut (E6) was combined with aneuronal bowel of 11 developmental days, quail cells were found at the sites of enteric ganglia in 7 of 11 cases (we produced this age-matched control by removing the hindgut from an chicken embryo at E4 and subsequent culturing for 7 days on the chorioallantoic membrane E4 + 7CAM). HNK-1, E/C8, and NF immunocytochemistry demonstrated that the quail cells indeed were neural crest cells or enteric neurons.

DISCUSSION

The development of the ENS provides an excellent model to study cell-cell interactions between neural crest cells and their target tissues. The present data show that quail neural crest cells in primitive enteric ganglia (E6 to E8) but not in older enteric ganglia (E10) can colonize aneuronal segments of chick gut. Furthermore, we demonstrated that quail neural crest cells do not colonize neuronal embryonic bowel (at E11).

The observation that clusters of neural crest cells in just neuronal embryonic bowel colonize neighbouring aneuronal segments suggests the existence of diffusing factors that regulate cell adhesion and migration of enteric neural crest cells. There is no outgrowth of neural crest cells when neuronal quail gut is cultured alone on the chorioallantoic membrane. When grown together with aneuronal chick gut, a number of neural crest cells leave the neuronal quail gut and colonize the aneuronal chick gut. Therefore, we surmise that the aneuronal chick bowel produces diffusing factors that trigger the release of neural crest cells in the neuronal segments.

The existence of such diffusing factors can also be deduced from quail-chick chimera experiments in which the quail neural anlage is inserted into a chick host. Normally quail neural crest cells migrate according to the migratory pathways of the new environment; however, the only exception is the vagal neural crest.[22] Vagal neural crest cells that are grafted to the "adrenomedullary" neural crest (adjacent to somite 18 to 24) migrate to the developing bowel. Based on

this observation LeDouarin et al surmised that vagal neural crest cells differ from other neural crest cells in their reaction to diffusing factors produced by the developing gut.[22] The existence of the diffusing substances in the developing gut is also supported by the studies of Rothman et al,[25] who inserted embryonic bowel segments in a slit aside the neural tube. They found excessive proliferation of central neurons at the site of the tube close to the bowel. It could be that in Hirschsprung's disease such diffusing factors or their neural crest cell receptors are abnormal.

These putative factor(s) have a releasing effect on neural crest cells in neuronal quail bowel of 6, 7, and 8 days of development, but not on neuronal quail gut at E10. We can only speculate about the reasons why quail neural crest cells cannot leave enteric ganglia at E10: (1) the degree of cell adhesion, (2) the existence of basal laminae surrounding the enteric ganglia, (3) the arrest of neural crest cell proliferation, (4) the acquisition of a neuronal phenotype, or (5) the absence of undifferentiated cells in enteric ganglia.

The observations that quail neural crest cells were present neither in the enteric ganglia nor in the surrounding regions of neuronal chick bowel, implies that quail neural crest cells do not colonize neuronal bowel. This observation points at an interesting phenomenon concerning the migration and homing of neural crest cells in the target organ. Previously, we reported that neural crest cells still can colonize aneuronal bowel segments even after aneuronal bowel has been cultured for 1 or 2 weeks on the chorioallantoic membrane.[17] Neural crest cell migration does not occur at the axial level after neural crest cells have used the migratory substrates or due to the disappearance of cell free spaces, but in the developing bowel, the migratory substrates remain present at least for 7 to 14 days. The migratory pathways in the embryo have been described in detail as relatively cell-free spaces lined by extracellular matrix molecules such as fibronectin, laminin, tenascin, and cytotactin. Tucker et al did not find such cell-free spaces in the developing chick bowel.[10]

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Regional differences between various axial segments of the avian neural crest regarding the formation of enteric ganglia

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Abstract. The vagal neural crest adjacent to somites 1–7 gives rise to the enteric ganglia along the entire digestive tract. It is generally assumed that formation of enteric ganglia in preumbilical gut is independent of the axial segment from which the neural crest originates. In postumbilical gut, however, there is evidence that the axial segment of origin of the neural crest might be relevant to neural differentiation. In this part of the gut, we previously identified a subpopulation of HNK-1-immunoreactive cells within the enteric mesenchyme. This immunoreactivity disappeared upon formation of the enteric nervous system. We studied the interaction between various axial segments of quail neural crest and the microenvironment in aneural chicken hindgut using chorioallantoic membrane cocultures. We found that neural crest cells from various axial segments could migrate into the gut and home to the correct sites. However, whereas vagal neural crest cells differentiated into enteric neurons, neural crest cells from truncal segments mainly differentiated into melanocytes. The HNK-1-immunoreactivity within the enteric mesenchyme only disappeared when neural crest cell colonization was followed by differentiation into enteric neurons and subsequent formation of enteric ganglia. To determine whether differentiation of neural crest cells in chorioallantoic membrane cocultures was influenced by the prolonged presence of the neural tube and notochord, we developed a new coculture system, using neural crest cells cultured in vitro. We found that the differentiation of vagal and trunk neural crest cells within the enteric mesenchyme was not influenced by the prolonged presence of the neural tube and notochord after 24 h, suggesting that there are intrinsic differences between these neural crest cell populations. Upon prolonged in vitro culturing, the properties of vagal neural crest cells changed, and concomitantly, they lost the ability to differentiate into enteric neurons and instead differentiated into melanocytes. We conclude that the disappearance of the HNK-1-immunoreactivity within the enteric mesenchyme is correlated with formation of enteric ganglia. In our experimental system, cells from the vagal neural crest are more capable of neural differentiation in the hindgut than cells from other axial levels of the neural crest.

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Introduction

The neural crest in vertebrate embryos is a transient structure extending along virtually the entire neural axis. Cells from the neural crest become widely distributed throughout the embryo and eventually localize at specific sites and differentiate into various cell types [4, 9, 16, 24, 25]. The vagal neural crest adjacent to somites 1–7 gives rise to mesectodermal and ganglionic derivatives. Mesectodermal derivatives, such as cells in the cardiac outflow tract, and the mesenchymal component of the thymus and the parathyroids, derive from a specific axial segment of the vagal neural crest from the level of the otic placode down to the third somite [3, 13, 14, 19]. Ganglionic derivatives of the vagal neural crest are the enteric ganglia [2, 17, 38], and dorsal root ganglia. Dorsal root ganglia are formed only by the vagal neural crest adjacent to somites 6 and 7, and not by crest adjacent to somites 1–5 [33].

It has been established that the neurons and supportive cells of the enteric nervous system along the entire digestive tract derive from the vagal neural crest [2, 17, 38]. Enteric ganglia in the postumbilical gut also receive a contribution from the sacral neural crest (caudal to somite 28), but its precise fate has not yet been established [17, 26, 27, 30]. A number of experimental studies have shown that neural crest other than vagal or sacral is able to colonize the gut [18, 23, 32, 34]. Le Douarin and Teillet [18] made quail-chick chimeras, in which the adrenomedullary neural crest adjacent to somites 18–24

was grafted to the vagal region of the neuraxis, and found quail cells along the entire digestive tract. In the preumbilical gut, these quail cells had differentiated into enteric neurons. Caudad to the umbilicus, however, some quail cells were found in the wall of the gut but they were exclusively pigment cells and never participated in ganglion formation. In chorioallantoic membrane cocultures of aneural chicken hindgut and adrenomedullary neural crest, enteric ganglia were observed, but the presence of melanocytes in the chicken hindgut was also reported [18, 23, 32, 34]. These melanocytes were located on either side of the smooth muscle layer, where plexus formation normally occurs. Thus, whereas after colonization of the preumbilical gut all trunk neural crest cells differentiate into enteric neurons, colonization of the postumbilical gut results in melanocyte differentiation of some trunk neural crest cells.

In a previous study, we cultured aneural chicken hindgut on the chorioallantoic membrane for 7 days and found a subpopulation of cells within the enteric mesenchyme that stained with the monoclonal antibody HNK-1 [20]. This HNK-1-immunoreactivity was localized in the submucosa just underneath the circular smooth muscle layer and between the circular and longitudinal smooth muscle layer, that is at the sites where ganglion formation normally occurs. This pattern of immunoreactivity in aneural gut, which we called HNK-1 mode 1, disappeared upon formation of the enteric nervous system. In neural gut the HNK-1 antibody marked the enteric ganglia, a pattern of immunoreactivity which we called HNK-1 mode 2. The HNK-1 antibody recognizes a carbohydrate epitope present on a large number of molecules all said to be involved in cell adhesion [11, 15]. Therefore, we speculated that these HNK-1-immunoreactive mesenchymal cells had some role in the homing of neural crest cells into the hindgut and/or their differentiation into enteric neurons. In this study, we further investigated this putative role.

We studied the interaction between various axial segments of the quail neural crest and the microenvironment of chicken aneural hindgut by performing chorioallantoic membrane cocultures. We confirmed that neural crest cells from quail vagal neural anlage differentiated into enteric neurons. Crest cells from trunk neural anlage migrated into the gut and homed to the sites of the myenteric and the submucous plexus, but in our experimental system they mainly differentiated into melanocytes. The HNK-1 immunoreactivity within the enteric mesenchyme only disappeared when crest-cell colonization was followed by the formation of enteric ganglia. To determine whether differentiation of neural crest cells in our cocultures was influenced by the prolonged presence of the neural tube and notochord, we developed a coculture system, in which we used neural crest cells cultured in vitro and demonstrated an intrinsic difference between vagal and trunk neural crest cells.

Methods

Animals. Fertilized chicken (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs were incubated at 38° C in a forced-draught humidified incubator. Embryos were staged according to the table of Hamburger and Hamilton [10]. We used quail neural primordia and chicken hindgut in our coculture experiments. The quail condensed heterochromatin marker was used to detect the presence of cells derived from quail neural crest in the gut [17].

Colonization assay. A 1-mm segment of hindgut just distal to the caecal bulges was isolated from 4-day-old chicken embryos (E4; HH stage 22-23). At this developmental stage, this part of the hindgut does not contain either vagal or sacral neural crest cells [20, 27, 34]. Neural anlagen, including neural crest, neural tube and notochord, were isolated from 2-day-old (E2) quail embryos. Vagal neural anlage adjacent to the first seven somites was isolated from embryos having 12–18 somites (HH stage 11–13). For trunk neural anlage, the crest adjacent to the last 6 somites was isolated from embryos having 16–34 somites (HH stage 12–16). The anlagen were dissected and freed of somites using a microscalpel. No digestive enzymes were used. The excised neural anlage was placed on an Immobilon P filter (Schleicher and Schuell, FRG) together with the segment of hindgut. This filter was then placed upside down on the chorioallantoic membrane of a 7-day-old chicken embryo and cultured for 7 days, to allow quail neural crest cells to colonize the chicken hindgut.

In vitro culture of neural crest cells. Neural anlagen were isolated from 2-day-old quail embryos as described above and placed on a glass coverslip coated with plasma fibronectin (100 µg/ml; obtained from human plasma using Sephadex-gelatin). The culture medium consisted of a 1:1 mixture of DMEM (Dulbecco's modified Eagle's medium, Flow Laboratories, UK) and F10 medium (Gibco, USA), 15% fetal calf serum (Sanbio, NL), 3% chicken embryo extract (prepared from 11-day-old embryos), glutamine (2.92 mg/ml), penicillin (0.75 mg/ml) and streptomycin (1.25 mg/ml). After 24 h the neural tubes were scraped off using a tungsten needle. The culture medium was changed twice a week. After a culture period varying from 1 day to 3 weeks, cultures were fixed and prepared for immunocytochemistry.

Colonization assay with neural crest cells cultured in vitro. After 16–20 h of culture the neural anlagen were scraped off using a tungsten needle. Neural crest cells were harvested with trypsin, EDTA (0.05%/0.02% in phosphate-buffered saline (PBS); w/w/v). Ten thousand neural crest cells were seeded onto an Immobilon P filter. After 30 min the cells had attached to the filter and a 1-mm segment of E4 chicken hindgut taken just distal from the caecal bulges was placed on top of this Immobilon P filter. Then the filter was placed upside down on the chorioallantoic membrane of a 7-day-old chicken host embryo and cultured for 7 days.

Immunohistochemistry. Transplants were harvested after the culture period and fixed in 2% paraformaldehyde in PBS for 24 h at room temperature. They were embedded in paraffin and sectioned at 7 µm. Cell cultures were washed twice in PBS and fixed in 2% paraformaldehyde in PBS for 30 min at room temperature. We used the monoclonal antibody HNK-1 as primary antibody [1] (ATCC; TIB 200; hybridoma supernatant, undiluted), incubated for 1 h at room temperature. Rabbit-anti-mouse immunoglobulins coupled to FITC (diluted 1:20; Dakopatts, Denmark) or rabbit-anti-mouse immunoglobulins coupled to horseradish peroxidase (diluted 1:100; Dakopatts, Denmark) were used as second-step antibodies. PBS containing 0.1% Tween-20 was used for all rinsing. Peroxidase was visualized with 0.1% 3,3'diaminobenzidine HCl (Serva, FRG)/0.01% H_2O_2 in PBS. Endogenous peroxidases were inhibited by a 20-min incubation in methanol/hydrogen peroxide (99:1 v/v) solution. Sections were counterstained with haematoxylin for 1 min. To visualize the quail condensed heterochromatin marker, sections were incubated with Hoechst 33258 (2 µg/ml PBS) for 4 min. For immunofluorescence, sections were analyzed using a Leitz orthoplan fluorescence microscope. Photographs were taken with a Leitz 35-mm camera using Kodak Ektachrome film (400 ISO). For immunoperoxidase, sections were analyzed using a Leitz

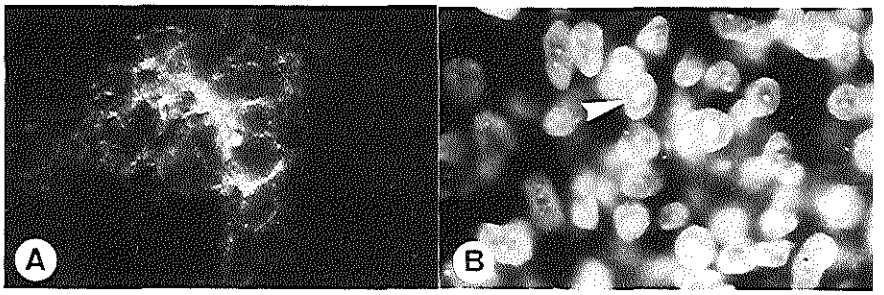

Fig. 1A, B. Paraffin section of a 7-day coculture of E2 quail vagal neural anlage and E4 chicken hindgut. A Immunofluorescence staining with the HNK-1 antibody showing a myenteric plexus. B Double-staining with Hoechst 33258 showing the presence of quail cells within this myenteric plexus (*arrowheads*). × 100

Orthoplan microscope and photographs were taken with a Leitz 35-mm camera using Agfa Ortho film (25 ISO) and a Kodak Wratten 49B filter.

Results

Neural crest cells from various axial segments colonize E4 chicken hindgut, but only vagal neural crest cells form enteric ganglia

Vagal neural anlage. We performed cocultures of quail vagal neural anlage and E4 chicken hindgut on the chorioallantoic membrane ($n = 8$). We observed clusters of quail cells in the gut at the site of both the myenteric and the submucous plexus as shown with the quail heterochromatin marker visualized with Hoechst 33258 (Fig. 1). In all cocultures with vagal neural anlage, the HNK-1 antibody revealed enteric ganglia, consisting of quail cells (Fig. 2A). The enteric mesenchyme showed no HNK-1 immunoreactivity.

Trunk neural anlage. Neural anlage adjacent to the last 6–8 somites was isolated from quail embryos with 16–34 somites and cocultured with E4 hindgut on the chorioallantoic membrane. This included neural crest from different axial segments in the trunk region, including adrenomedullary crest. These different trunk crest segments gave identical results. After 7 days of coculture with E4 hindgut, the HNK-1 antibody did not reveal enteric ganglia ($n = 8$). Instead, we still observed HNK-1 mode 1 immunoreactivity within the enteric mesenchyme, localized at the site of the myenteric and the submucous plexus. We observed numerous HNK-1-negative melanocytes at the sites of the myenteric and the submucous plexus and a few HNK-1-immunoreactive quail cells, which could have been enteric neurons (Fig. 2B, C). These HNK-1-immunoreactive quail cells, however, were dispersed and had not formed enteric ganglia. It was difficult to determine the species origin of the melanocytes, because of the large number of pigment granules present in the cells. Clear quail origin was demonstrated in about 10% of the melanocytes. In control cultures of aneural hindgut without neural crest cells, melanocytes were never observed ($n = 20$; data not shown).

These results confirm that vagal neural anlage is capable of forming enteric ganglia in the postumbilical gut. Trunk neural crest cells are able to migrate into the gut and colonize the gut at the appropriate sites, but they are not able to form enteric ganglia, and mainly differentiate into melanocytes. HNK-1 mode 1 immunoreactivity only disappeared when crest cell colonization was followed by the formation of enteric ganglia.

Differentiation within the enteric microenvironment is determined by intrinsic properties of vagal and trunk neural crest cells and not influenced by the prolonged presence of the neural tube and notochord

We studied whether the variation in differentiation observed between vagal and trunk neural anlage was influenced by the prolonged presence of the neural tube and notochord during the colonization assay, or based on an intrinsic difference between vagal and trunk neural crest cells. We developed a new coculture system, in which neural crest cells were allowed to migrate out of the neural tube in vitro. After 24 h the neural crest cells were collected, seeded onto an Immobilon P filter, and cocultured with a segment of chicken E4 hindgut on the chorioallantoic membrane. After 7 days of coculture, we found that quail vagal neural crest cells, cultured for 1 day in vitro, were still capable of forming enteric ganglia ($n = 6$). The enteric ganglia, however, were somewhat smaller and fewer than cocultures with vagal neural anlage. Staining with the HNK-1 antibody revealed enteric ganglia (HNK-1 mode 2), although there was still some HNK-1-immunoreactivity present within the enteric mesenchyme (Fig. 3A). A minimum of ten thousand vagal neural crest cells per filter, equivalent to the outgrowth of 5–10 neural anlagen, was necessary to obtain colonization of the gut. The same number of cultured neural crest cells from trunk colonized the gut at the

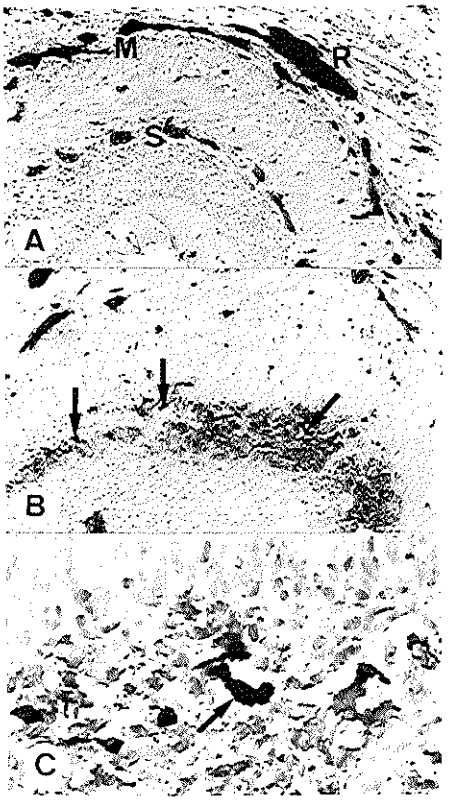

Fig. 2A–C. Paraffin sections of 7-day cocultures of E2 quail neural anlage and E4 chicken hindgut. A Vagal neural anlage: staining with the HNK-1 antibody shows mode 2, visualizing the myenteric (*M*) and the submucous (*S*) ganglia. Remak's ganglion (*R*) is also stained. ×40 B Trunk neural anlage: staining with the HNK-1 antibody shows HNK-1 mode 1-immunoreactivity, visualizing a layer of immunoreactive cells within the enteric mesenchyme at the site of the submucous plexuses. Within this layer melanocytes are present (*arrows*). ×40 C Detail of B showing melanocytes (*arrow*). ×63

proper sites but did not give rise to enteric ganglia ($n=3$). Instead we observed melanocytes and more-intense HNK-1 mode 1 immunoreactivity (Fig. 3B). Thus, the difference in differentiation seen earlier between vagal and trunk neural anlage was based on an intrinsic difference between vagal and trunk neural crest cells. The prolonged presence of the neural tube and notochord after 24 h had no effect on neural crest cell differentiation.

Intrinsic differences between vagal and trunk neural crest cells can be illustrated in vitro

Some characteristics of vagal and trunk neural crest cells were studied in vitro. First, vagal and trunk neural crest cells were compared after 1 day of in vitro culture, at the time vagal neural crest cells were capable of forming enteric ganglia in the colonization assay. Vagal neural crest cell cultures consisted of about one to two thousand small flattened stellate cells. After fixation and staining with the HNK-1 antibody, we observed 40%–50% HNK-1 positive cells (based on the counts of six cultures; Fig. 4A). Trunk neural crest cell cultures consisted of one to two thousand cells, which were small and round to stellate. These cultures consisted of a higher percentage (75%–80%) of HNK-1 positive cells (based on the counts of five cultures; Fig. 4B). Occasionally, small clusters of epithelial-like cells were observed in both vagal and trunk neural crest cell cultures (Fig. 4A, arrow). These could have been remnants of ectoderm or the neural tube. We then studied vagal and trunk neural crest cells upon further culturing. During the first week of culture, vagal neural crest cultures showed moderate proliferation (determined by the increase in cell number; data not shown). The percentage of HNK-1-positive cells rapidly declined during the first 4 days of culture, from 40%–50% on day 1 to 5%–10% on day 4, and remained low during the next week of culture. Trunk neural crest cells proliferated rapidly, showing a vast increase in cell number (data not shown). During the first week of culture, the percentage of small stellate HNK-1-positive cells remained high (85%–90%). After the first week it gradually declined, to about 20% after 3 weeks. In Fig. 5 the changes in the percentage of HNK-1-positive cells during culture of vagal and trunk neural crest cells are shown.

The differences in morphology and HNK-1 expression between vagal and trunk neural crest cells after 1 day of culture in vitro illustrate an intrinsic difference between these two cell populations. The decline in HNK-1 expression of vagal neural crest cells upon prolonged in vitro culturing indicates that vagal neural crest cell properties change during in vitro culture.

In vitro changes of vagal neural crest cell properties affect their differentiation in the hindgut

To investigate whether the changes of vagal neural crest cell properties observed during in vitro culture influenced their differentiation behaviour, we tested vagal neural crest cells, cultured for 4 days in vitro, in our colonization assay ($n=5$). We found that vagal neural crest cells were still able to colonize the gut at the proper sites, but they were no longer able to differentiate into enteric neurons. Instead, melanocytes developed in the gut (Fig. 6A). Trunk neural crest cells cultured for 4 days still gave rise to melanocytes ($n=3$; Fig. 6B).

These data further support the hypothesis that there is an intrinsic difference between vagal and trunk neural crest cells just after migration from the neural anlage.

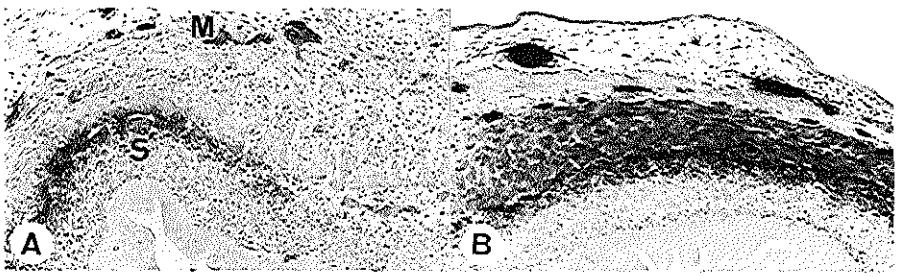

Fig. 3A, B. Paraffin sections of 7-day cocultures of quail vagal neural crest cells, cultured for 1 day in vitro, and E4 chicken hindgut. A Vagal neural crest cells: the HNK-1 antibody visualizes myenteric (*M*) and submucous (*S*) plexuses. There is still some HNK-1 mode 1-immunoreactivity visible. ×40 B Trunk neural crest cells: the HNK-1 antibody visualizes mode 1-immunoreactivity, and no enteric neurons are present. ×40

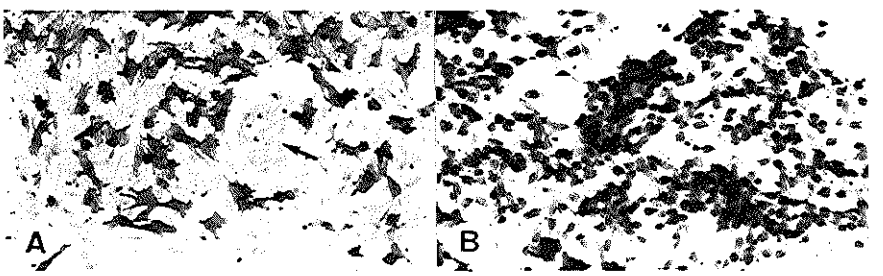

Fig. 4A, B. Quail neural crest cells cultured for 1 day in vitro. A Vagal neural crest cells: staining with HNK-1 reveals about 40%–50% of HNK-1-immunoreactive cells, which are of a stellate, flattened morphology. The small cluster of epithelial-like cells present in the culture (*arrow*) is probably a neural tube remnant. B Trunk neural crest cells: Staining with HNK-1 reveals a high percentage (75%–80%) of HNK-1-immunoreactive cells of a small, round to stellate morphology. ×40

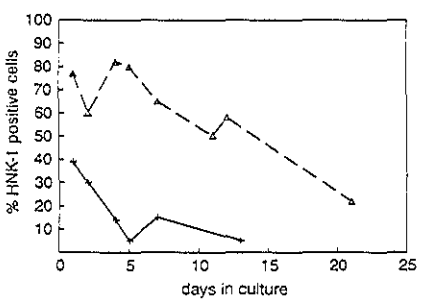

Fig. 5. Changes in the percentage of HNK-1-expressing cells in neural crest cell cultures. The *solid line* represents vagal neural crest cells, the *dashed line* represents trunk neural crest cells

The specific characteristics of vagal neural crest cells which enable them to form enteric ganglia in the postumbilical gut are lost upon prolonged culturing in vitro. They retain their colonization properties, but they now differentiate similarly to trunk neural crest cells.

Discussion

Differentiation of vagal and trunk neural crest in postumbilical gut

Neural crest from both vagal and trunk levels could colonize the hindgut, but under our experimental conditions, formation of enteric ganglia was confined to vagal neural crest cells. Trunk neural crest cells migrated and homed to the correct sites but they mainly differentiated into melanocytes. Neural differentiation sometimes occurred, but enteric ganglia did not develop. This implies that neural crest cells from various axial levels are able to recognize migration and homing signals in the enteric microenvironment, but that cells from the vagal neural crest are better capable of responding correctly to differentiation signals.

Le Douarin and Teillet [18] introduced the chorioallantoic membrane coculture method as an assay for neural crest cell colonization of the hindgut and in this way established pluripotentiality of the neural crest. They showed that trunk neural crest cells can give rise to enteric ganglia, and this method was subsequently used in a number of studies [23, 32, 34]. All those studies de-

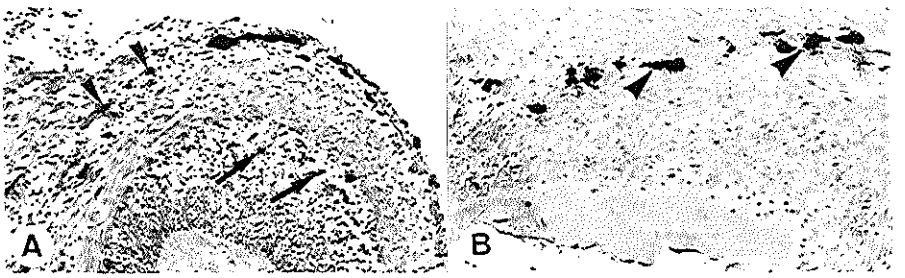

Fig. 6A, B. Paraffin sections of 7-day cocultures of E2 quail neural crest cells, cultured for 4 days in vitro, and E4 chicken hindgut. A Vagal neural crest cells: Many melanocytes are in the submucosa (*arrows*) and there are als some melanocytes at the site of the myenteric plexus (*arrowheads*). × 40 B Trunk neural crest cells: there are many melanocytes in the submucosa (*arrowheads*). × 40

scribed enteric ganglia in the hindgut, but the number of ganglia varied, possibly in relation to the experimental system used. A near to normal number of enteric ganglia was found, when E4.5 or E5 chicken hindgut was cocultured with E2 quail neural anlage, which was introduced into a slit made in the hindgut and subsequently cultured for 12–24 h in vitro before placing it on the chorioallantoic membrane [18, 32, 34]. In direct cocultures of E4 hindgut and E2 neural anlage, without making a slit and omitting the in vitro culture, enteric neurons were extremely sparse with many sections not containing neurons [23]. Smith et al. [32] attributed this difference between vagal and trunk neural crest segments to a quantitative difference between these two neural crest regions, the vagal neural crest being larger than trunk neural crest. We found, however, that an amount of vagal neural crest equivalent to the length of two somites is already sufficient for the formation of a normal enteric nervous system in this coculture system (unpublished results). All these coculture studies agree on the presence of melanocytes in the hindgut after colonization with trunk neural crest, which is in line with our results. The capacity of trunk neural crest cells to give rise to enteric neurons was also tested in heterotopic quail-chick chimera in which the adrenomedullary crest was transplanted to the vagal level. In the preumbilical gut, trunk neural crest gave rise to a normal enteric nervous system, whereas in the postumbilical gut the quail cells found in the gut wall were exclusively pigment cells and never participated in ganglion formation [18]. Additional evidence for a specific interaction between vagal neural crest cells and the gut comes from the same study, in which quail vagal neural crest was transplanted to the adrenomedullary region of a chicken embryo [18]. The vagal neural crest behaved as adrenomedullary crest except that in the gut there were quail cells, which had differentiated into enteric neurons. In normal development trunk neural crest cells never penetrate the splanchnic mesoderm, indicating that at this level no preferential route leads the cells to the intestine [18]. These data indicate that although trunk neural crest cells can give rise to enteric neurons under certain experimental conditions, they differ from vagal neural crest cells regarding their differentiation into melanocytes in the postumbilical gut.

The role of the enteric microenvironment in the formation of the enteric nervous system

In this study we found that the HNK-1-immunoreactivity within the enteric mesenchyme of aneural gut disappeared only when colonization by neural crest cells was followed by the formation of enteric ganglia. After colonization with trunk neural crest the HNK-1-immunoreactivity persisted within the enteric mesenchyme. Thus the switch from mode 1 to mode 2 HNK-1-immunoreactivity correlates with the differentiation of neural crest cells into enteric neurons and the subsequent formation of enteric ganglia, and not with mere colonization. These results indicate that there is an interaction between neural crest cells and the enteric microenvironment.

A number of studies describe an influence of the enteric microenvironment of neural crest cell differentiation. Dulac and Le Douarin [5] found that the Schwann cell marker SMP on enteric glial cells is down-regulated by the influence of the gut wall. Pomeranz et al. [28] suggested that an interaction between laminin in the enteric microenvironment and a laminin binding protein on neural crest cells plays a role in the homing of neural crest cells in the gut and the subsequent development of enteric ganglia. Galliot et al. [7] described expression of the *Hox-1.4* gene in fetal mouse gut mesenchyme during normal embryonic development. Wolgemuth et al. [37] made transgenic mice overexpressing the *Hox-1.4* gene and found that this resulted in abnormal gut development. It was found that overexpression of *Hox-1.4* resulted in a nonfunctional enteric nervous system, because the neural crest cells differentiated into catecholaminergic neurons, displaying the ultrastructure of peripheral, not enteric, nerves [8].

Considering these results, it is possible that the enteric microenvironment in the postumbilical gut produces a signal necessary for the differentiation of neural crest cells into enteric neurons. Vagal neural crest cells would then respond to this signal and differentiate into enteric

neurons, whereas trunk neural crest cells are less able to recognize this signal or react to it in a different way.

Intrinsic difference between vagal and trunk neural crest cells illustrated in vitro

We developed a new coculture system, which uses in vitro cultured neural crest cells, and demonstrated that the prolonged presence of the neural tube and notochord after 24 h did not influence the difference in neural versus melanocyte differentiation between vagal and trunk neural anlage in the hindgut. In the absence of the neural tube, a minimum of ten thousand vagal neural crest cells, cultured for 1 day, was needed to achieve colonization and to form enteric ganglia. The enteric ganglia, however, were somewhat smaller and fewer than with vagal neural anlage, and there was still some HNK-1 mode 1-immunoreactivity present. During the formation of enteric ganglia, HNK-1 mode 1 immunoreactivity gradually disappeared. In chorioallantoic membrane cocultures using vagal neural anlage, HNK-1 mode 1 had disappeared by the 7th culture day (personal observation). Using cultured neural crest cells could cause a delayed or incomplete disappearance of HNK-1 mode 1 immunoreactivity. This could be related to the number of neural crest cells used, or to a change in vagal neural crest cell properties even during the first day of in vitro culture.

We attempted to correlate the observed difference in differentiation in vivo with an intrinsic difference between vagal and trunk neural crest cells in vitro. We found that 40%–50% of the vagal neural crest cells showed HNK-1-immunoreactivity after 1 day of in vitro culture, whereas trunk neural crest consisted of 75%–80% HNK-1-immunoreactive cells. There are a number of studies on the expression of the HNK-1 epitope on trunk neural crest cells in vitro [22, 29, 31, 36]. Our result of 75%–80% HNK-1-positive trunk neural crest cells after 1 day of culturing agrees with three other studies reporting the percentage of HNK-1-positive cells after 1 day of culture [22, 29, 35]. In these studies there are no data on the percentage of HNK-1-immunoreactive cells in vagal neural crest cell cultures. It is not clear whether the observed difference in HNK-1 expression between vagal and trunk neural crest cells in vitro reflects a similar difference in vivo. Up to now the HNK-1 antibody has been the only marker for early migrating neural crest cells in chicken embryos and therefore it was not possible to detect HNK-1-negative neural crest cells in vivo. With the recently developed lineage tracer techniques, such as DiI and retroviral markers [6, 21, 27], it will be possible to study neural crest cell development in vivo independent of HNK-1-immunoreactivity.

On prolonged culture, the percentage of HNK-1-immunoreactive vagal neural crest cells rapidly declined to 5% during the next 3 days in vitro, indicating a change in characteristics of these cultured cells. Vincent and Thiery [36] showed that a number of neural crest derivatives lose their HNK-1 epitope and that only neural crest derivatives related to the peripheral nervous system retain this epitope. The observed drop in percentage of HNK-1-immunoreactive cells is concomitant with a loss of capability to differentiate into enteric neurons. Whereas after 1 day of culture vagal neural crest cells showed normal colonization and differentiation properties, after 4 days of culture they lost the ability to form enteric neurons and instead differentiated into melanocytes.

In conclusion, we surmise that the formation of the enteric nervous system in the postumbilical gut entails at least two phases. First, neural crest cells migrate through the gut and home to the sites of the myenteric and the submucous plexus. This colonization phase is not specific for vagal neural crest cells: crest cells from other axial segments are also able to colonize the gut. During the second phase, neural crest cells differentiate into enteric neurons and form enteric ganglia, for which the enteric microenvironment probably provides a signal. Correct differentiation into enteric neurons depends on intrinsic properties of vagal neural crest cells. In our experimental system, most trunk neural crest cells either cannot recognize the signal or react to it differently and differentiate into melanocytes. The switch from mode 1 to mode 2 HNK-1 immunoreactivity occurs upon formation of enteric ganglia, during the second phase.

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Characterization of HNK-1 antigens during the formation of the avian enteric nervous system

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Summary

During vertebrate embryogenesis, interaction between neural crest cells and the enteric mesenchyme gives rise to the development of the enteric nervous system. In birds, monoclonal antibody HNK-1 is a marker for neural crest cells from the entire rostrocaudal axis. In this study, we aimed to characterize the HNK-1 carrying cells and antigen(s) during the formation of the enteric nervous system in the hindgut. Immunohistological findings showed that HNK-1-positive mesenchymal cells are present in the gut prior to neural crest cell colonization. After neural crest cell colonization this cell type cannot be visualized anymore with the HNK-1 antibody. We characterized the HNK-1 antigens that are present before and after neural crest cell colonization of the hindgut. Immunoblot analysis of plasma membranes from embryonic hindgut revealed a wide array of HNK-1-carrying glycoproteins. We found that two HNK-1 antigens are present in E4 hindgut prior to neural crest cell colonization and that the expression of these antigens disappears after neural crest colonization. These two membrane glycoproteins, G-42 and G-44, have relative molecular masses of 42,000 and 44,000, respectively, and they both have isoelectric points of 5.5 under reducing conditions. We suggest that these HNK-1 antigens and the HNK-1-positive mesenchymal cells have some role in the formation of the enteric nervous system.

Key words: HNK-1, neural crest, cell adhesion molecule, enteric nervous system.

Introduction

Glycosylation and sulphatation are important post-translational modifications of proteins serving essential roles in embryonic development (Sorkin et al., 1984; Feizi, 1985; Imamura and Mitsui, 1987). Thorpe and coworkers (1988) suggested that carbohydrate structures play crucial roles in intercellular interactions. All proteinaceous cell adhesion molecules described so far are glycoproteins, but the function of the carbohydrate moieties has only been elucidated in a few cases (Hoffman and Edelman, 1983; Sadoul et al., 1983). One particular carbohydrate moiety, a complex sulphate-3-glucuronyl residue, is recognized by monoclonal antibody HNK-1. Chou and coworkers (1985, 1986) characterized the molecular structure of the HNK-1 epitope both in a glycolipid and in a tetrasaccharide which had been isolated from human peripheral nerves. It is likely that the carbohydrate moiety on glycoproteins is similar if not identical to the epitope on the glycolipid and the tetrasaccharide (Shashoua et al., 1986; Burger et al., 1990). The HNK-1 epitope is present on a series of molecules involved in cell adhesion, substratum adhesion and extracellular matrix interactions (Kruse et al., 1984; Faissner, 1987; Pesheva et al., 1987; Hoffman and Edelman, 1987). Keilhauer and coworkers (1985) and Künemund and coworkers (1988) demonstrated that the HNK-1 epitope itself is involved in neuron-neuron and glial-glial cell interactions in in vitro adhesion assays. Bronner-Fraser (1987) showed that injection of the HNK-1 antibody lateral to the mesencephalic neural crest of chicken embryos even perturbs neural crest migration in vivo.

In chicken embryos, HNK-1 antigens are present at very early stages of development (Canning and Stern, 1988). Using immunoablation, Stern and Canning (1990) found that HNK-1-positive cells play a key role in gastrulation. During neurulation, HNK-1 visualizes premigratory and migrating neural crest cells. It is accepted that the HNK-1 antibody can be used as a marker for avian neural crest cells (Tucker et al., 1984; Vincent et al., 1983; Tucker et al., 1986; Newgreen et al. 1990), although structures not derived from the neural crest can also be HNK-1 positive (Serbedzija et

al., 1991). At later stages of development the HNK-1 monoclonal antibody visualizes rhombomeres three and five (Kuratani et al., 1991), and the central, peripheral and enteric nervous systems. The HNK-1 epitope has also been found in the nervous systems of other vertebrate and invertebrate species (Schwarting et al., 1987; Mikol et al., 1988; Tucker et al., 1988; Dennis et al., 1988; Poltorak et al., 1989; Nordlander, 1989; Metcalfe et al., 1990). Interspecies differences in HNK-1 expression in the brain have been reported (O'Shannessy et al., 1985; Holley and Yu, 1987). In addition, the expression of HNK-1 antigens in various structures changes during development (Mikol et al., 1988; Newgreen et al., 1990).

In the chicken embryo, the neurons and supportive cells of the enteric nervous system develop from rhombencephalic (vagal) neural crest cells, which emerge at the level of somites 1-7, and sacral neural crest cells, which emerge posterior to somite 28 (LeDouarin and Teillet, 1973). Using the HNK-1 antibody to visualize neural crest-derived cells in the wall of the gut, Epstein and coworkers (1991) found that crest-derived enteric precursors form a cellular network when they reach the primordial gut distal to the lung buds. The authors suggest that this network is probably a precursor to the ganglionic network in the adult proximal gut. The behaviour of the crest-derived cells, which underlies the formation of this network, is different from the behaviour of crest-derived populations forming autonomic and sensory ganglia. The microenvironment of the gut may be a major component in producing this different behaviour. There is no knowledge concerning the molecular nature of the HNK-1 antigens during the formation of the enteric nervous system.

In this study, we aimed to characterize the HNK-1-carrying cells and antigen(s) during the formation of the enteric nervous system in the hindgut. We therefore investigated explants of the hindgut at various stages of development with immunohistochemical and biochemical techniques.

Materials and methods

Embryos

Fertilized eggs of *Gallus gallus domesticus* were obtained from a local supplier and incubated in a forced draught incubator at 37°C and 80% humidity. Embryos were staged according to the number of incubational days (E=day of development) or to the table of Hamburger and Hamilton (1951).

Explantation of embryonic hindgut

Gut segments were isolated between the ceca and the cloaca. We used E4 till E14 embryos. The mesentery was removed.

Chorioallantoic membrane cultures

Segments of 1 mm hindgut were grafted onto the chorioallantoic membrane as described previously (Meijers et al., 1987). E4 hindgut was harvested after seven days and E7 hindgut after four days' culture. The chorioallantoic membranes and blood vessels were removed and the dissected grafts were homogenized or prepared for immunohistochemistry. For coculture experiments, the vagal neural primordia adjacent to the first seven somites of stage 10 embryos were dissected and cocultured with E4 and E7 hindgut for seven and four days, respectively.

Immunohistochemistry

After dissection, gut segments were rinsed in phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde-PBS, dehydrated and embedded in paraffin (Fluka, Switzerland). Sections were made at 5-7 μm. Alternatively, segments were embedded in Tissue Tek II embedding compound (Miles, Naperville, IL) and snap-frozen in liquid nitrogen-cooled isopentane. Sections were made at 10 μm. Sections were incubated with the primary antibody in a moist incubation chamber at room temperature for one hour. For immunofluorescence, rabbit anti-mouse FITC-conjugated $F(ab)_2$ fragments of immunoglobulins (Dako, Denmark) were used as a second step antibody (diluted 1:20). For immunoperoxidase staining, rabbit anti-mouse peroxidase-conjugated immunoglobulins (Dako, Denmark) were used as second step antibodies (diluted 1:100). Endogenous peroxidases were inhibited by a 20 minute incubation in methanol/hydrogen peroxide (99:1/ v/v) solution. Peroxidase was visualized with 0.1% 3,3'diaminobenzidine.HCl (Serva, FRG) with 0.01% hydrogen peroxide. Sections were counterstained with hematoxylin for one minute. PBS with 0.1% Tween-20 was used for all rinsing. Sections were evaluated using a Leitz Fluorplan microscope, or with a Biorad Confocal Laser Scanning Microscope mounted on a Nikon fluorescence microscope.

Antisera

The HNK-1 hybridoma was purchased from the American Tissue Type Culture Collection (TIB200) (Abo and Balch, 1981). Cells were grown in RPMI medium (Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum (Sanbio, Uden, the Netherlands), penicillin 0.75 mg/ml, streptomycin 1.25 mg/ml and glutamine 2.92 mg/ml (Life Technologies, Breda, the Netherlands). Conditioned media were harvested after three days' culture. Monoclonal antibody E/C8 was purchased from the Developmental Studies Hybridoma Bank. mAb E/C8 is directed against NAPA-73, a neurofilament-associated glycoprotein. The supernatant of the hybridoma culture was used undiluted (Ciment and Weston, 1982; Ciment et al., 1986).

Homogenisation of tissues for plasma membrane analysis

Plasma membranes were obtained according to a modification of the protocol of Maeda and coworkers (1983). In brief, tissues were collected in TSE buffer (10 mM Tris/HCl pH 8.0, 0.25 M sucrose, 1 mM EDTA) at 4°C, homogenized in an Omni Mixer Homogenizer (Connecticut, USA) on ice for 1 minute (level 10). The suspension was centrifuged for 5 minutes at 2000 revs/minute in an Heraeus centrifuge. The supernatant was layered to a solution containing 41% sucrose, 10 mM Tris/HCl pH 8.0, 1 mM EDTA, and centrifuged in a Beckman ultracentrifuge for 1 hour at 24.000 revs/minute in a SW28 rotor. The interphase containing the plasma membranes was collected and diluted with TSE buffer and centrifuged again for 1 hour at 24.000 revs/minute in a SW28 rotor. The pellet was resuspended in TSE buffer and stored at −70°C. Protein content was determined with the BCA assay (Pierce USA). We purified 105 μg plasma membrane protein from 350 explants of E4 gut (total protein content per explant

40 μg protein); 69 explants of E7 gut contained 1.4 mg plasma membrane protein; 10 explants of E14 gut contained 3.0 mg plasma membrane; 40 cultures of E4 hindgut contained 1.2 mg plasma membrane proteins; 50 cultures of E7 hindgut contained 0.54 mg plasma membranes.

Two-dimensional gel electrophoresis

Gels with a length of 6.5 cm were prepared in glass tubes with a diameter of 2 mm according to the manufacturer's description (Biorad, California). In brief, 0.25 ml Biolyte 3/10 ampholyte, 0.25 ml Biolyte 5/7 ampholyte, 2 ml 10% Triton X-100, 5.5 g urea analytical grade (Merck, FRG), 1.33 ml acrylamide solution (28.3% acrylamide and 1.62% piperazine di-acrylamide (Biorad, California), 1.97 ml distilled water, 10 μl 10% ammonium persulfate, 10 μl N,N,N',N'-tetra-methylethylenediamine (Biorad, California)) were mixed and allowed to polymerize in glass tubes at 37°C. Pre-electrophoresis was performed at 200, 300, 400 V for 10, 20 and 20 minutes, respectively.

For isoelectric focusing, samples were diluted with an equal volume of sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris/HCl pH 6.8, bromophenol blue, 0.1% dithiotreitol (Calbiochem, California), boiled for 3 minutes and chilled on ice. SDS was added to the protein sample to facilitate solubilization. Samples were then diluted with an equal volume of lysis buffer (9.5 M urea, 2% Triton X-100, 0.1% dithiotreitol, 1.6% Biolyte 5/7 ampholyte, 0.4% Biolyte 3/10 ampholyte in distilled water). The SDS was removed from the proteins by the Triton X-100 micelles. Samples of 100 μl containing 50 μg of protein were loaded under an overlay solution and subjected to electrophoresis for 3.5 hours at 600 V.

For separation in the second dimension, the gels were gently removed from the tubes and equilibrated for approximately 90 minutes in sample buffer until the pH indicator in the acid part of the gel became blue. The gel tubes were loaded directly on a 2.25 mm thick 7.5% SDS-polyacrylamide minigel (Biorad, California) and subjected to electrophoresis for 15 minutes at 100 V and then at 200 V until the bromophenol blue reached the bottom of the gel. Carbamylyte creatine phosphokinase (Pharmacia, Sweden) was used as a standard for isoelectric focusing and prestained protein molecular weight standards (14.3 - 200 × 10^3 M_r) (Bethesda Research Laboratories, MD) were used in the second dimension.

HNK-1 immunoblotting

Proteins were transferred from the gel onto a 0.45 μm nitrocellulose SSBA85 membrane (Schleicher and Schuell, FRG) in a Biorad blot apparatus at 200 mA and 100 V for 90 minutes in a blotting buffer containing 20% (v/v) methanol/50 mM Tris/Glycine pH 8.0. Blots were blocked by overnight incubation in 2% bovine serum albumin (Sigma, Fraction V, St. Louis) in PBS-Tween-20 (0.1%) at 4°C and 20 minutes incubation in 1% normal goat serum (Amersham International Plc, UK) in PBS-Tween-20 at room temperature. Subsequently, the blots were incubated with a twenty times diluted supernatant of HNK-1 hybridoma culture for 45 minutes at room temperature. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin IgM $F(ab)_2$, (Tago, Inc., Burlingame, USA), in a dilution of 1:10.000 in PBS-Tween-20, was used as second step antibody (incubation 45 minutes at room temperature). Between each incubation step the blots were rinsed five times with 50 ml PBS-Tween-20 (0.1%). Phosphatases were visualized using a protocol from Blake and coworkers (1988). For control immunoblots we used the unconditioned medium which was used to culture the HNK-1 hybridoma cells. All other steps were identical.

Results

HNK-1 immunoreactivity in the developing gut from E4 till E12

We determined the HNK-1 immunoreactivity in the normal developing hindgut during (E4-E6) and after neural crest cell migration and colonization (E7-E14).

At E4, there are few differentiated cell types present in the gut. The epithelium is multilayered and there are no layers of smooth muscle cells present. The enteric mesenchyme is surrounded by a thin layer of serosal cells. Proximal to the cecal bulges, HNK-1 immunoreactivity is present on the outer surface of serosal cells (Fig. 1). Underlying the serosal cells, HNK-1-positive cells are present within the mesenchyme. HNK-1 immunoreactivity is also present underneath the serosal cells in the most distal segment of the gut, but a 1 mm segment distal to the cecal bulges does not contain any HNK-1-positive cells.

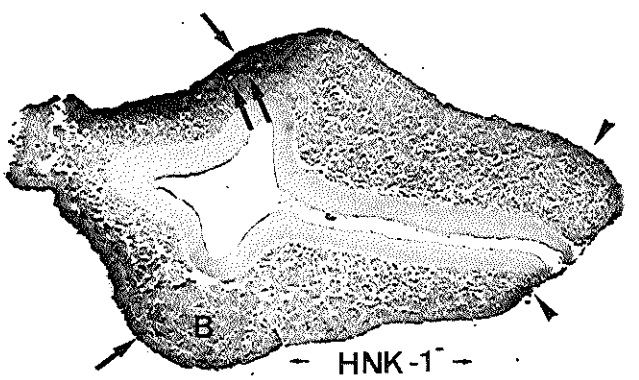


Fig. 1. Longitudinal paraffin section of the postumbilical E4 gut. Proximal to the cecal bulges (B), HNK-1 immunoreactivity is present on the outer cell membrane of serosal cells (single arrow) and in the mesenchyme underneath (double arrows). In the caudal gut, HNK-1 immunoreactivity is confined to cells in the serosa (arrowheads). The 1 mm gut segment distal to the cecal bulges does not contain HNK-1 immunoreactivity (HNK-1⁻). ×25.

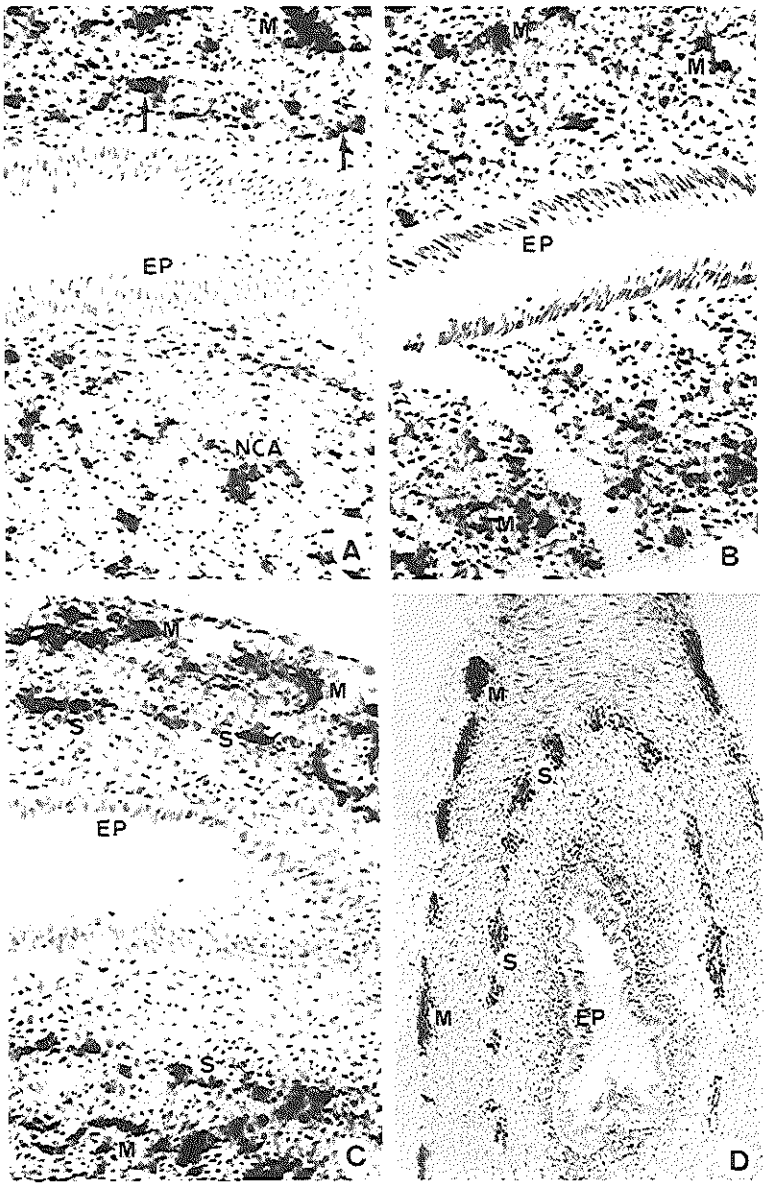


Fig. 2. HNK-1-stained cryostat sections of E5 (A), E6 (B), E7 (C) and E14 (D) hindgut segments. HNK-1 visualises the developing enteric ganglia. At E5 and E6, faint HNK-1 immunoreactivity is present between aggregates of neural crest cells (arrows). At E7, neural crest cell colonization of the (hind)gut is completed. At E14, HNK-1 immunoreactivity in the myenteric ganglia is stronger than in the submucous ganglia. Compared with the submucous plexus, the size of the myenteric ganglia has increased considerably. ep, epithelium; m, myenteric plexus; nca, neural crest aggregate; s, submucous plexus. Magnification 25×.

At E5, HNK-1 immunoperoxidase staining of cryostat sections revealed some clustered positive cells in the mesenchyme of the periumbilical gut (Fig. 2A). In addition to the heavily stained cell membranes of these clustered cells, we observed a weaker and disperse staining in the mesenchyme.

At E6, clusters of HNK-1-positive cells are located at the sites of the myenteric and submucous ganglia (Fig. 2B). As in the E5 gut, we observed a disperse and fainter HNK-1 staining between the developing submucous ganglia.

At E7, the clustering of positive cells is more pronounced and one can distinguish between the myenteric and submucous ganglia (Fig. 2C). The mesenchyme was HNK-1 negative.

At E14, HNK-1 immunoperoxidase staining revealed the relatively large myenteric plexus, located between the thin longitudinal and the circular smooth muscle layer, and the relatively small submucous plexus, at the luminal side of the circular smooth muscle layer (Fig. 2D). The HNK-1 immunoreactivity was located at the cell membrane of enteric neurons and their processes (immunofluorescence data not shown). The smooth muscle cells and the mesenchyme in the submucosa were not stained by the HNK-1 antibody. Sometimes the apical site of the epithelium was HNK-1 positive.

Four low relative molecular mass HNK-1-carrying plasma membrane glycoproteins are present in E4 but not in E7 and E14 hindgut

To determine the molecular nature of the HNK-1 antigens in the hindgut during the formation of the enteric nervous system, we produced two-dimensional gels for immunoblot analysis. In the plasma membrane fraction of E4 hindgut, we observed spots with relative molecular masses of 200, 130, 44, 42, 27 (doublet) and 20 (doublet) $\times 10^3$ (Fig. 3A). The spot at 42×10^3 had a lower intensity than the 44×10^3 spot. The HNK-1 glycoproteins of 200 and 130×10^3 represent various glycoproteins with different isoelectric points (pI range from 7.1 to 5.5). The HNK-1 glycoproteins of relative molecular masses of 44, 42, 27 and 20×10^3 are single spots. The isoelectric points of these proteins are 5.5, 5.5, and for both doublets 5.5 and 4.9, respectively. We did not observe any spot in control immunoblots.

In immunoblots of the plasma membrane fraction of E7 hindgut, we only observed intense spots in the high relative molecular mass range (200, 110-130 $\times 10^3$) (Fig. 3B). The isoelectric points of these high relative molecular mass glycoproteins ranged from 7.1 to 6.5 for the 200×10^3 protein, and from 7.5 to 5.5 for the 110-130 $\times 10^3$ glycoproteins. A characteristic "orion-like" pattern of low intensity HNK-1 glycoproteins was found in the low molecular range (M_r/pI: A, 50×10^3/5.0; B, 40×10^3/5.4; C, 30×10^3/5.8; D, 29×10^3/6.4) facilitating the identification of other low relative molecular mass glycoproteins. We did not observe the

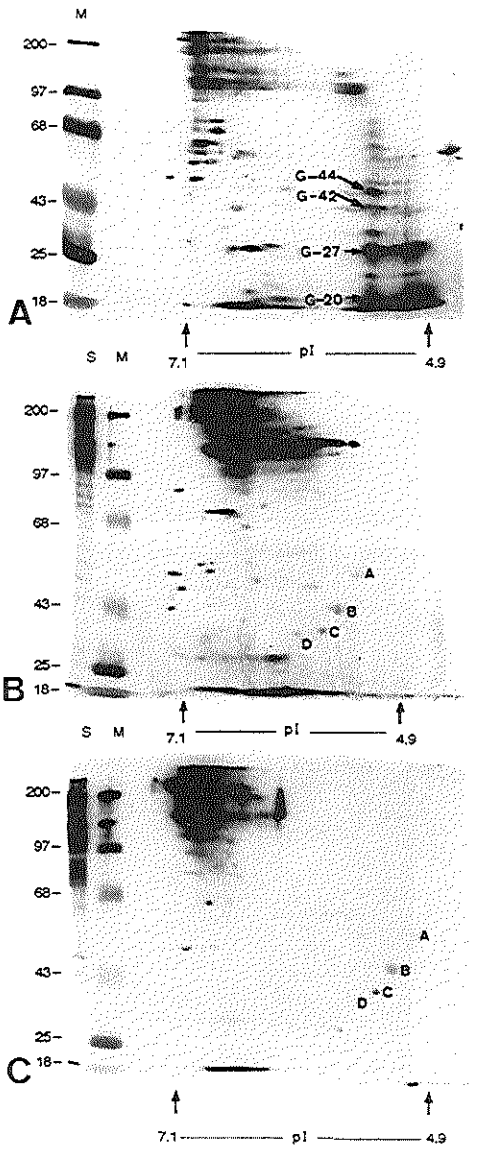
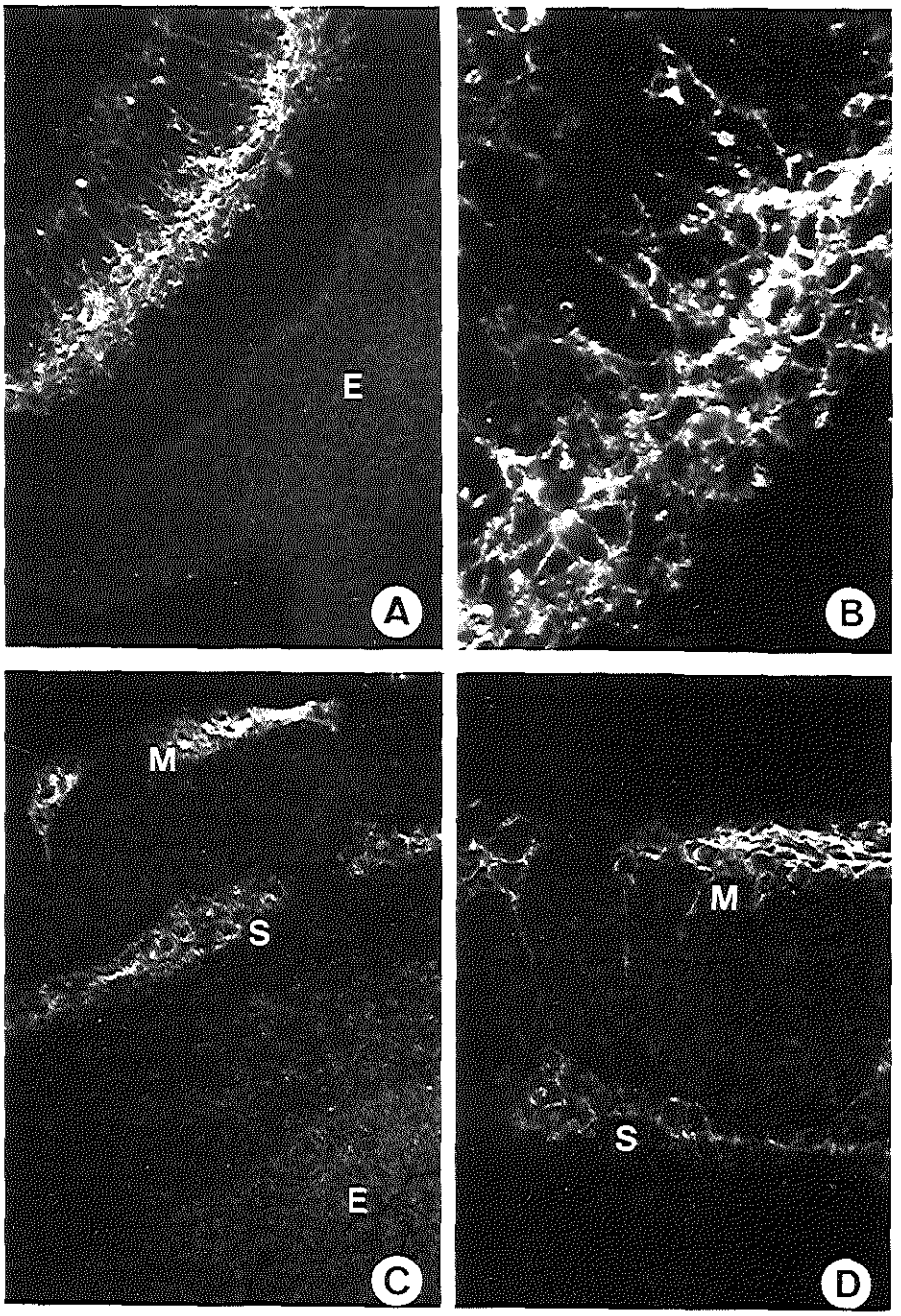


Fig. 3. Two-dimensional HNK-1 immunoblots of plasma membranes isolated from different embryonal stages of the gut (A, E4; B, E7; C, E14). Note the presence of low M_r HNK-1 antigens (G-44; G-42; G-27; G-20) in E4 hindgut and the absence of these molecules in E7 and E14 hindgut. HNK-1 antigens in the high relative molecular mass region (200 and 110-130 $\times 10^3$) are present both in E4 and E7/E14 hindgut. M, high molecular weight markers; S, plasma membrane starting material. The isoelectric focusing range was determined by Carbamolyte markers.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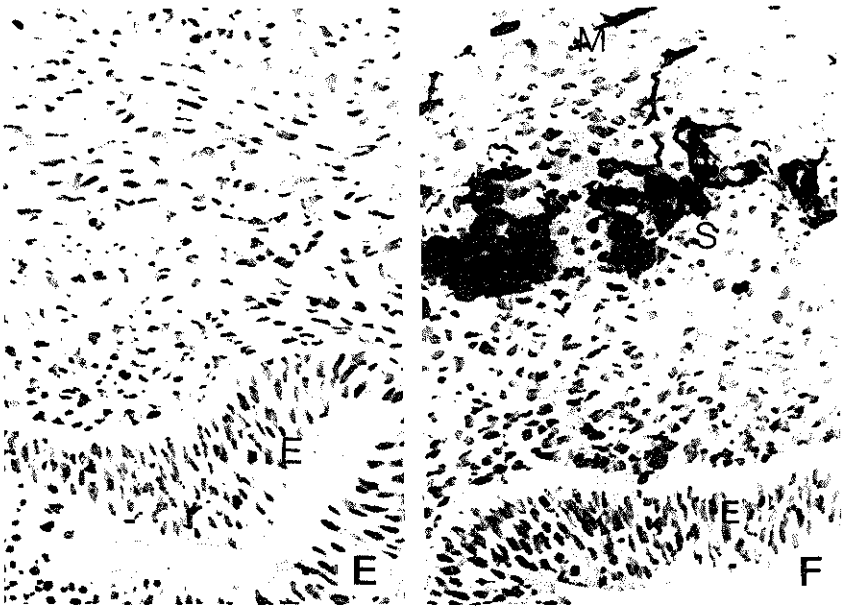

Fig. 4. (A) Confocal laser scanning image of cultured E4 hindgut. A layer of HNK-1 positive mesenchymal cells inside circular smooth muscle layer is present (HNK-1 mode 1). ×16. (B) Detail of A. ×40. (C) Confocal laser scanning image of a coculture of E4 hindgut and the neural primordium. HNK-1-positive neural crest cells or enteric neurons are present in enteric ganglia (HNK-1 mode 2). Note the absence of the layer of HNK-1-positive mesenchymal cells. ×16. (D) Detail of C. The image was made at a different level from that in C. ×25. (E) Cryostat section of cultured E4 hindgut stained with the neuron specific antibody (E/C8). Note the absence of immunoreactivity. (F) Cryostat section of cultured E4 hindgut and vagal neural primordium stained with E/C8. Note the strong immunoreactivity in the submucous plexus. ×25. E, epithelium; M, myenteric plexus; S, submucous plexus.

44, 42, 27 and 20 × 10^3 HNK-1 glycoproteins present in E4 hindgut.

In two-dimensional HNK-1 immunoblots of the plasma membrane fraction of E14 hindgut we observed intense spots in the high relative molecular mass range (200, and 110-130 × 10^3) (Fig. 3C). The isoelectric points of the 200 × 10^3 protein ranged from 7.1 to 6.5 and for the 110-130 × 10^3 proteins the pI ranged from 7.1 to 6. The characteristic orion-like pattern of single HNK-1 glycoproteins (A-D) was identical to that in E7 gut. The HNK-1 immunoblot findings are summarized in Table 1. From these results, we conclude that E4 hindgut contains low relative molecular mass HNK-1-positive glycoproteins (G-44, G-42, G-27, G-20) which are not present in E7 and E14 hindgut.

HNK-1 immunoreactivity in chorioallantoic cultures of embryonic gut

To test whether the disappearance of low relative molecular mass HNK-1 antigens correlates with neural crest cell colonization of the gut, we cultured E4 hindgut without and with the vagal neural primordium of stage HH 10 embryos. After the culture, we characterized the HNK-1-positive cell types and the HNK-1-carrying antigens.

Confocal laser scanning microscopy revealed that HNK-1-positive cells are present in cultured E4 hindgut. These cells are located in (a) a circular layer of cells in the submucosa at the luminal side of the circular smooth muscle layer, and (b) in spots between the longitudinal and circular smooth muscle layer (Fig. 4A and B). The HNK-1-immunoreactive cells in the submucosa were connected to the HNK-1-positive spots at the site of the myenteric plexus by HNK-1-positive fibers. We will refer to this pattern of HNK-1 immunoreactivity in cultured E4 hindgut as HNK-1 mode 1 immunoreactivity. It is important to stress that the HNK-1-positive cells in the submucosa develop in cultures of gut segments that do not contain neural crest cells or any other HNK-1-positive cell types at the time of explantation.

Coculture of E4 hindgut and the neural primordium resulted in an apparently normal enteric nervous system in the transplant (see also LeDouarin and Teillet, 1973;

Table 1. *HNK-1 antigens ($M_r \times 10^3$) in plasma membranes of hindgut*

Aganglionic hindgut		Ganglionic hindgut				
Normal E4	Cultured E4	Cocultured E4+NP	Normal E7	Cultured E7	Cocultured E7+NC	Normal E14
200	200	200	200	200	200	200
130	130	110-130	110-130	110-130	130	130
44	44	–	–	–	–	–
42	42	–	–	–	–	–
37	–	–	–	–	–	–
20	–	–	–	–	–	–
–	mode 1	mode 2	mode 2	mode 2	mode 2	mode 2

E, day of development; NP, neural primordium; Mode 1 and 2, type of HNK-1 immunoreactivity (see text).

Allan and Newgreen, 1980). The neurons and the neurites in the enteric ganglia were HNK-1 positive (Fig. 4C and D). We will refer to this immunohistological pattern of HNK-1 immunoreactivity as HNK-1 mode 2. Culture of E7 hindgut also resulted in HNK-1 mode 2 staining.

In quail-chick chimeras, sacral neural crest cells migrate along the dorsal surface of the gut and give rise to the ganglion of Remak during E4 through E6 (LeDouarin and Teillet, 1973). To exclude that the HNK-1 mode 1 staining in cultured E4 hindgut is due to neural crest cells that have migrated from Remak's ganglion through the serosa, we dissected the E5 hindgut and removed Remak's ganglion (it was not possible to remove Remak's ganglion from E4 hindgut). After one week of culture, HNK-1 visualized the layer of cells in the submucosa, HNK-1 mode 1 (data not shown). Thus HNK-1 mode 1 is not due to sacral neural crest cells that have migrated from Remak's ganglion.

To investigate whether the HNK-1 mode 1 cells have neuronal characteristics, we performed immunohistochemistry on cryostat sections with the E/C8 antibody (Fig. 4E). We did not find E/C8-positive cells or cells with a neuronal phenotype in the cultured E4 hindgut. In addition, there were no neurofilament-positive cells in cultures of E4 hindgut (data not shown). In contrast, HNK-1 mode 2 coincided with E/C8-positive cells in the enteric ganglia (Fig. 4F). HNK-1 mode 1 did not coincide with immunoreactivity with antibodies specific for three characterized HNK-1 antigens (N-CAM, chicken integrin, tenascin; data not shown). We determined which segments of the gut exhibit HNK-1 mode 1 by culturing successive segments of postcecal bowel of E5, E6 and E7 gut. As is shown in Fig. 5, HNK-1 mode 1 reactivity is present in cultures of the most distal gut of E4 through E6. By E7 all cultures of the gut show HNK-1 mode 2 immunoreactivity. The cultures of the distal E4 through E6 gut did not contain enteric ganglia, thereby representing aganglionic gut. Therefore, HNK-1 mode 1 immunoreactivity is confined to aganglionic segments of the gut, while HNK-1 mode 2 immunoreactivity is related to ganglionic gut segments.

Two low relative molecular mass HNK-1 carrying plasma-membrane glycoproteins are present in cultures of E4 gut but not in (co)cultures of E7 gut and neural primordium

We cultured explants of E4, E7 hindgut and cocultured E4 hindgut and the neural primordium until both types of explants had reached the age of 11 developmental days.

In immunoblots of the plasma membrane fraction of cultured E4 hindgut we observed HNK-1-positive spots with relative molecular masses of 200, 130, 44 and 42 × 10^3 (Fig. 6A). The range of isoelectric points of these glycoproteins is similar to that observed for HNK-1 antigens in explanted E4 hindgut. The orion-like distribution of HNK-1 antigens A through D facilitated the proper identification of HNK-1 antigens in the

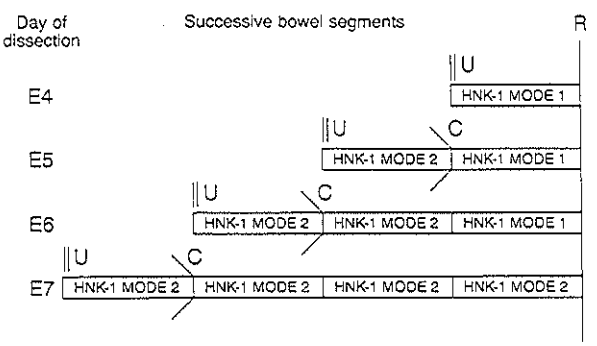

Fig. 5. HNK-1 reactivity in cultures of successive segments of postcecal bowel of E5, E6 and E7 gut. R, rectum; C, cecum; U, umbilicus.

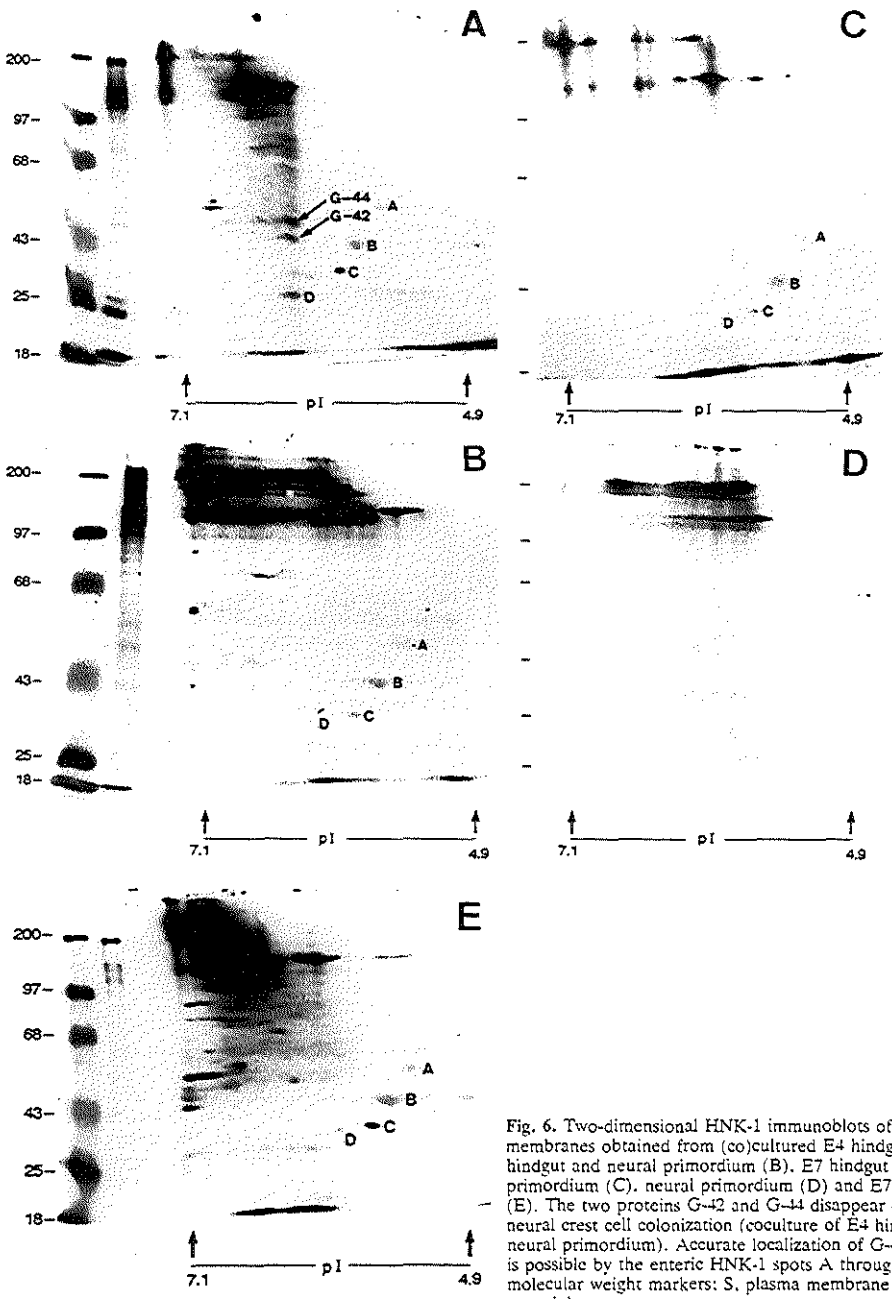

Fig. 6. Two-dimensional HNK-1 immunoblots of plasma membranes obtained from (co)cultured E4 hindgut (A), E4 hindgut and neural primordium (B), E7 hindgut and neural primordium (C), neural primordium (D) and E7 hindgut (E). The two proteins G-42 and G-44 disappear during neural crest cell colonization (coculture of E4 hindgut and neural primordium). Accurate localization of G-42 and G-44 is possible by the enteric HNK-1 spots A through D. M, molecular weight markers; S, plasma membrane starting material.

lower molecular range. The doublets at 27 and 20 × 10^3 had disappeared. But the HNK-1 glycoproteins of 44 and 42 × 10^3 were still present.

In immunoblots of the plasma membrane fraction of cocultures of E4 hindgut with the neural primordium, we observed intense spots in the high relative molecular mass range (200, 130-110 × 10^3) (Fig. 6B). The orion-like distribution of HNK-1 antigens A through D was also present. The G-42 and G-44 HNK-1 glycoproteins were absent. Thus during the coculture either the expression of the HNK-1 epitope on G-42 and G-44 or the expression of these HNK-1-bearing proteins disappeared. Neural crest cell colonization did not yield additional HNK-1-positive spots.

To test which HNK-1 glycoproteins are expressed by the neural primordium, we cultured it in combination with E7 hindgut or alone (Fig. 6C and D). In immunoblots of the plasma membrane fraction of cultures of neural primordium, we observed HNK-1-positive bands in the high relative molecular mass range (200, 130-110 × 10^3) (Fig. 6D). The orion-like distribution of HNK-1 antigens A through D that is normally seen in plasma membrane fractions of the gut was not observed. Furthermore, the G-42 and G-44 were not detected.

In immunoblots of the plasma membrane fraction of cultured E7 hindgut, we observed a string of intense spots in the high M_r range (200, 110-130 × 10^3) (Fig. 6E). In the low M_r range, we observed HNK-1 antigens A through D. The HNK-1-positive spots G-42 and G-44 seen in (cultured) explants of E4 hindgut were absent. In cocultures of E7 hindgut and the neural primordium, we found a similar picture to that found in cultures of E7 hindgut alone (Fig. 6C and E). The HNK-1 antigens in plasma membranes of (co)cultured E4 and E7 hindgut are summarized in Table 1.

From these experiments, we conclude that G-42 and G-44 are present in (cultured) explants of E4 hindgut and absent in (cultured) explants of E7 hindgut. The spatiotemporal expression of G-42 and G-44 coincides with neural crest cell colonization in the hindgut.

Discussion

HNK-1-positive enteric mesenchyme and the formation of enteric ganglia in the postumbilical gut

HNK-1 immunostaining of E4 hindgut shows that HNK-1-positive cells are located anterior to the cecal bulges and in the most distal colorectum. The anterior HNK-1-positive cells could reflect the vanguard of migrating vagal neural crest cells. However, the presence of neural crest cells just proximal to the cecal bulges does not correspond with the time of arrival of neural crest cells that has been reported in earlier studies (LeDouarin and Teillet, 1973; Allan and Newgreen, 1980). According to these authors, vagal neural crest cells migrate in the preumbilical gut at E4 (stage 24). They reach the umbilical region by E5, and the cecal region by E6. The presence of HNK-1-positive cells in the most distal segment of the hindgut at E4 is in agreement with the studies of Pomeranz and Gershon (1990, 1991).

We found that HNK-1-positive cells develop in cultures of HNK-1 negative explants of E4 hindgut. These HNK-1-positive cells are distributed as a mesenchymal cell layer at the luminal side of the circular smooth muscle layer (HNK-1 mode 1). Thus, even if HNK-1-positive vagal and sacral neural crest cells are not present within a hindgut-segment, HNK-1-positive cells still develop. This suggests that the HNK-1-positive cells in HNK-1 mode 1 do not derive from the neural crest. The existence of HNK-1-negative neural crest cells in stage 24 gut is an objection to this assumption. However, this is unlikely since it is generally accepted that the majority of sacral neural crest cells are HNK-1 positive (Pomeranz and Gershon, 1990). In view of the distribution of the HNK-1-positive mesenchymal cells in the submucosa and in the myenteric region, we surmise a splanchnopleural mesoderm origin. An endodermal origin for these cells is not likely but cannot be excluded.

The HNK-1-positive mesenchyme in cultured E4 hindgut seems to be organized in a network. The submucosal layer of HNK-1-positive cells is connected to the HNK-1-positive cells in the myenteric region by HNK-1-positive tracts. Epstein and coworkers (1991) observed a neural crest-derived HNK-1 positve network in the foregut which initiates the formation of the enteric nervous system. We found a HNK-1-positive network in the hindgut which does not derive from the neural crest. It could well be that the formation of the enteric nervous system in the foregut and hindgut may be mediated by different mechanisms.

New members of the L2/HNK-1 family

Although the HNK-1 epitope is present on a family of cell adhesion molecules, it is surprising that a wide array of HNK-1 antigens is present in the developing gut at particular developmental stages. Most of the cloned members of the L2/HNK-1 family of cell adhesion molecules have relative molecular masses over 100 × 10^3. The high M_r HNK-1 antigens in the plasma membranes of the developing gut could be known members of the HNK-1 family of adhesion molecules such as the neural cell adhesion molecule N-CAM (M_r=200, 180, 160, 140 × 10^3), the β subunit of the fibronectin receptor and the laminin receptor (M_r=135 × 10^3).

HNK-1 antigens with low relative molecular masses (<100 × 10^3) are more abundant at early embryonic stages compared to later stages (Canning and Stern, 1988). We detected low relative molecular mass HNK-1 antigens in immunoblots of two-dimensional gels of plasma membranes of early (E4) embryonic gut (G-44, G-42, G-27 and G-20). The HNK-1 family of adhesion molecules has grown substantially and several of the cDNAs encoding for the protein backbones have been cloned and sequenced. Only two members of the HNK-1 family with relative molecular masses lower than 100 × 10^3 have been identified: the myelin protein P_0 (M_r=19.6-26.5 × 10^3) (Lemke et al., 1988) and an

acetyl-cholinesterase of *Electrophorus* electric organs (M_r approximately 70 × 10^3) (Bon et al., 1987). Thus the relative molecular masses of the HNK-1 antigens G-42 and G-44 do not resemble known HNK-1 antigens. There is little knowledge about the proteins that carry the HNK-1 epitope during early vertebrate development. However, most, if not all, members of the known members of the L2/HNK-1 family play a role in cell adhesion. We surmize that G-42 and G-44 represent two unidentified HNK-1-carrying cell adhesion molecules.

G-42 and G-44 in E4 hindgut disappear during neural crest cell colonization

Neural crest cell colonization of the hindgut occurs during E4 through E7. We detected low M_r HNK-1 antigens in the gut prior to neural crest cell colonization. Two of these, G-20 and G-27, are present in explants of E4 hindgut and disappear during culture. G-42 and G-44 proteins are present prior to neural crest cell colonization but they disappear after neural crest cell colonization, both in vivo and in cocultures.

An important issue to resolve is which HNK-1 antigens cause HNK-1 mode 1. Due to the lack of additional markers for G-42 and G-44 we cannot ascribe HNK-1 mode 1 exclusively to these two proteins.

In sections of cultured E4 hindgut most of the HNK-1 immunoreactivity is located in HNK-1 mode 1, whereas in immunoblots of similar cultures the most prominent HNK-1-positive spots are found in the relative molecular mass range of 200 and 110-130 × 10^3. Because high relative molecular mass HNK-1 antigens are present in (cultured) E7 and E14 gut and in cultures of the neural primordium, it is not unlikely that they represent neuronal antigens. The presence of these neuronal HNK-1 antigens in E4 hindgut can be ascribed to the presence of extrinsic nerve fibres or Remak's ganglion. Another possibility is that the HNK-1-positive mesenchymal cells contain these neuronal HNK-1 antigens and that the expression continues after neural crest cell colonization.

HNK-1-positive cells and antigens are essential for gastrulation and the development of the mesencephalic neural crest in chicken embryos (Stern and Canning, 1990; Bronner-Fraser, 1987). These findings, taken together with the adhesive characteristics of HNK-1 antigens, suggest that the HNK-1 antigens in E4 hindgut might play a role in adhesive or in repulsive interactions with enteric neural crest cells. As such, the HNK-1-positive mesenchymal cells might play a role in the initiation of the patterning of the enteric nervous system in the hindgut.

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CHAPTER 3.7.

Pattern of malformations and dysmorphisms associated with Hirschsprung disease: an evaluation of 214 patients

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Abstract

Several lines of evidence suggest the involvement of genetic factors in the pathogenesis of Hirschsprung disease. As an initial step to identify these factors we determined the incidence and nature of associated anomalies both in long-segment and short-segment Hirschsprung disease. We retrospectively examined the charts of 214 patients with Hirschsprung disease admitted to the Sophia Children's Hospital between 1970 and 1992. We found that short-segment Hirschsprung disease was present in 170 cases (♂ : ♀, 4 : 1), long-segment in 44 (♂ : ♀, 3:1). The overall incidence of associated anomalies was 27.6%. We found associated anomalies in 39 patients with short-segment (22.9%) and in 20 patients with long-segment disease (45.5%). We distinguished five classes of Hirschsprung disease depending on the presence and nature of the associated anomalies. The first class entailed isolated cases of Hirschsprung disease. The ♂ : ♀ ratio was 4.7 : 1, with no difference between short- and long-segment disease. The second class entailed cases with Down syndrome (7.6%); these patients had short-segment disease and were predominantly males. The third class entailed 'syndromic' cases, the incidence of which was highest in long-segment disease (20.5% versus 5.9% in short-segment disease). The ♂ : ♀ ratio in 'syndromic' cases was 1 : 1. In the fourth class Hirschsprung disease was associated with craniofacial dysmorphisms, and in the fifth class with one other anatomic malformation. We conclude that short-segment and long-segment Hirschsprung disease do not differ markedly with respect to the pattern of associated anomalies, sex ratio, and familial occurrence. It might well be that isolated forms of short- and long segment disease are variant forms of the same genetic defect. The association with Down syndrome seems to be confined to short segment Hirschsprung disease.

Introduction

Hirschsprung disease (HSCR) is characterized by the absence of enteric neurons and the presence of hypertrophic nerve trunks in the distal digestive tract. Over the last 30 years, many case reports and several large cohort studies have established clinical, pathological and genetic heterogeneity for HSCR. A positive family history has been described in approximately 7% of all cases [Kleinhaus et al., 1979]. Genetic study of HSCR has proven difficult, because of the limited availability of large pedigrees. Until fairly recently, patients with HSCR hardly gave rise to offspring. The high number of sporadic cases and the fact that the disease is four times more frequent in boys than in girls suggested a sex-modified multifactorial mode of inheritance, involving multiple genes. Badner et al. [1990], however, showed that, while for short segment (SS)-HSCR the inheritance pattern was equally likely to be either multifactorial or due to a recessive gene with low penetrance, for long segment (LS)-HSCR the mode of inheritance was most compatible with an autosomal dominant gene with incomplete penetrance. HSCR may be associated with other malformations, but the reported incidence ranges from 5.3% to 29.8 [Ehrenpreis, 1970, Spouge and Baird, 1985, Ikeda and Goto, 1986]. Many of these studies were limited by the small size of the populations studied, and attention was mainly focused on major malformations that required surgical repair, not on detailed dysmorphologic description of HSCR patients. Several of the combinations of HSCR and associated anomalies acquired a McKusick index number, representing Mendelian Inheritance in Man (for OMIM numbers see Table I).

Well-defined patient populations are a prerequisite to study congenital malformations

Table I: McKusick Index numbers of Congenital Malformations of the Enteric Nervous System

1	Hirschsprung disease (HSCR)	249200
2	HSCR with ulnar polydactyly, polysyndactyly of the big toes,VSD	235750
3	HSCR with type D brachydactyly	306980
4	HSCR and Ondine's curse	209880
5	HSCR and microcephaly and iris coloboma	235730
6	HSCR with pigmentary anomaly	277580
7	HSCR and hypoplastic nails and dysmorphic features	235760
8	HSCR and Waardenburg syndrome type 1	193500
9	HSCR and polydactyly and renal agenesis	235740
10	Total intestinal aganglionosis	202550
11	Intestinal pseudo-obstruction	243180

using molecular genetic techniques. As an initial step to study possible pathogenetic mechanisms of one or more varieties of intestinal aganglionosis, we determined the sex-ratio and the incidence of associated malformations and dysmorphisms in a large population of HSCR patients, and characterized these. We wondered whether we could distinguish SS- and LS-HSCR based on these parameters. Establishing the presence of associated malformations might help in syndrome delineation. Even clinically insignificant anomalies may bear major information value for diagnostic and epidemiologic purposes.

Patients and methods

We collected the files of all 227 patients with histologically proven HSCR admitted to the Sophia Children's Hospital between 1970-1992. The data abstracted from the files included: (1) sex; (2) length of aganglionic segment (pathology reports); (3) presence of associated malformations and/or dysmorphisms; (4) presence of a family history of aganglionosis or other congenital malformations; (5) causes of death in the 17 patients who died. Not all parameters were available in each file. In 13 patients, we were not able to trace the length of the aganglionic segment. These patients were excluded from further analysis. A questionnaire was sent to patients admitted between 1970-1985 (patients indicated with A in tables), asking for familial HSCR and for associated anomalies in the patient. From 1986 till 1992 (patients indicated with B in tables), 33 of 59 patients had been evaluated by a dysmorphologist.

Definitions: First we classified the patients according to the length of the aganglionic segment, measured from the internal anal sphincter. We will refer to HSCR involving anus, rectum and part of the sigmoid colon as SS-HSCR. Long-segment HSCR entails the colon proximal to the sigmoid colon, whereas total colonic aganglionosis (Zuelzer Wilson disease) involves the entire colon. Sometimes the terminal ileum is also involved, but the aganglionosis rarely involves the entire gut caudal to the duodenum (total intestinal aganglionosis). We will refer to long-segment HSCR, total colonic aganglionosis, and total intestinal aganglionosis collectively as LS-HSCR.

We defined major and minor malformations as anatomic abnormalities that are present at birth (e.g. pulmonary artery stenosis, renal agenesis, or Meckel's diverticulum). We also included disorders such as psychomotor retardation, deafness, and central hypoventilation as malformations in our analysis. Dysmorphisms are defined as developmental deviations from the usual morphological form, with an antenatal origin. The term anomalies will be used to indicate both malformations and dysmorphisms. Patients with only one minor malformation or dysmorphism, such as a sacral dimple or abnormal handcreases, were scored as having no associated anomalies.

Statistical analysis was performed using the X^2 test, using SPSS software.

Results

SS-HSCR was most common (n = 170, 79.4%), while LS-HSCR was present in 44 cases (20.6%) (including 11 cases with total colonic aganglionosis and 3 cases with total intestinal aganglionosis). We did not ascertain cases with ultrashort aganglionosis or skip lesions. Of the 170 SS-HSCR patients, 136 were male and 34 were female (♂ : ♀, 4 : 1). The ♂ to ♀ ratio

for LS-HSCR was 3 to 1 (33 males to 11 females). The distribution of the different lengths of the aganglionic segment and the sex ratio are depicted in Figure 1. These two parameters are in agreement with frequencies reported in other series [Kleinhaus et al., 1979, Ikeda and Goto, 1984]. This indicates that our patient population is a representative group of cases with intestinal aganglionosis.

The overall incidence of associated anomalies was 27.6%. In our A series (admitted between 1970-1985), the incidence of associated anomalies was 19.8%, and in our B series (admitted between 1985-1992) 48.3%. This difference in incidence is statistically significant ($p < 0.001$)

Fifteen of the 214 patients died (7%). Eight patients died due to complications of aganglionosis (including 3 patients with total intestinal aganglionosis), and 6 patients died due to associated malformations. Among these 6 patients were 2 patients with Down syndrome, who died due to cardiac failure. In one case the cause of death was not known (Table II). These figures indicate that the presence of associated malformations is a significant cause of death.

Short-segment HSCR

In the majority of patients SS-HSCR was the only malformation (n = 131; 77.1%). Associated malformations were present in 39 patients (22.9%). We divided these 39 patients into four additional classes according to the nature of the associated malformation(s). The incidence

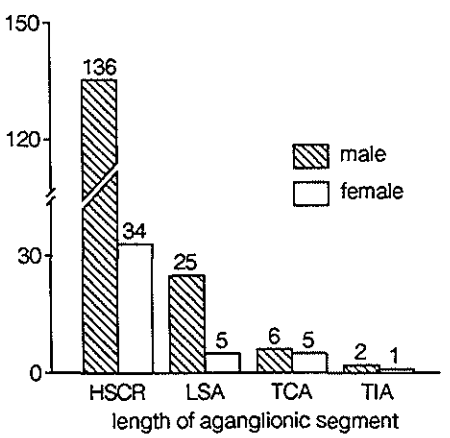

Figure 1: Length of the aganglionic segments. Diagram depicting the percentages of cases with classic Hirschsprung's disease (HSCR), long segment aganglionosis (LSA), total colonic, and total intestinal aganglionosis (TCA and TIA) and the sex distribution within each class.

Table II: Causes of death in patients with intestinal aganglionosis

Death related to the intestinal aganglionosis:

Patient	Sex	Cause
A41	M	Enterocolitis, sepsis
A132	M	Suture leakage, sepsis, multiple organ failure
A131	M	Postoperative bowel perforation, sepsis
A17	M	Suture leakage, sepsis
A45	F	Necrotic bowel, septic shock
B32	M	Total intestinal aganglionosis
B5	M	Total intestinal aganglionosis
A23	F	Total intestinal aganglionosis

Death caused by associated malformations

Patient	Sex	Cause
A49	M	Respiratory tract infection, atrio-ventriculo septal defect, mitral valve insuffiency, aortic coarctation, Down's syndrome
B37	M	Complications during surgery of atrio-ventriculo septal defect, Down's syndrome
A51	M	Blalock shunt dysfunction in patient with atrio-ventriculo septal defect, atresia of tricuspid valve
B54	F	Sepsis in patient with multiple congenital malformations
B45	F	Disseminated neuroblastoma, Ondine's curse
B42	F	Sepsis, ventilatory complications, Ondine's curse

Patient	Sex	Cause
A150	M	Unknown

and sex ratios of the various classes of SS-HSCR are summarized in Table III. Class II entails 13 cases with Down syndrome (7.6%). Of these 11 were male and 2 were female. Eight of these 13 patients also had cardiac malformations. All other SS-HSCR patients with associated anomalies are presented in Table IV. Class III entails 10 other 'syndromic' cases (5.9%; ♂

to ♀., 1 to 1). These patients had both major and minor malformations and craniofacial dysmorphisms. A syndrome diagnosis was generally not made in these patients. Patient B42 had Ondine's curse (OMIM 209880). Two patients (A152 and A3) had mental retardation and epilepsy. Limb abnormalities were present in patients A18 and A48. Three patients (A48, A24, and A135) had congenital deafness. Class IV entails 4 patients with craniofacial dysmorphisms only (2.3%; ♂ to ♀, 3 to 1). Facial dysmorphism included broad nasal bridge, antimongoloid eyes, and dysplastic and low set ears.

Class V entails 12 patients with one other major/minor malformation in addition to HSCR (7.1%; ♂ to ♀ 5 to 1). Patient B35 had a pulmonary artery stenosis. Five patients had a Meckel's diverticulum. Patient B49 also had a Meckel's diverticulum, but we considered this as part of a 'syndrome', so this patient was ranked class III. Patient B53 had polydactyly and rocker bottom feet. Three patients showed vesico-ureteral reflux, of which one also had hydronephrosis (A72).

We identified 5 families that presented 14 cases with isolated HSCR (♂ to ♀, 7 : 4; for the other 3 cases the sex was unknown). All HSCR families are presented in Table V. We identified 4 families in which SS-HSCR was associated with other malformations. These families provided 6 cases of SS-HSCR, one case with LS-HSCR and two cases with unknown

Table III: Incidence and sex-ratio of the various classes of both short- and long-segment HSCR

		Short-segment HSCR			Long-segment HSCR		
Class		n	%	♂:♀	n	%	♂:♀
	Total	170		4:1	44		3:1
I	No associated anomalies	131	77.1	4.5:1	24	54.5	7:1
II	Down syndrome	13	7.6	5.5:1	1	2.2	
III	'Syndromic'	10	5.9	1:1	9	20.5	5:4
IV	Associated with cranio-facial anomalies	4	2.3	3:1	5	11.4	2:3
V	Associated with other anomalies	12	7.1	5:1	5	11.4	4:1

length of the aganglionic segment (♂ to ♀, 8 to 1). In proband B50, SS-HSCR was associated with craniofacial dysmorphisms and nystagmus. A brother of this patient's father had isolated HSCR. In patient A114, SS-HSCR was associated with Meckel's diverticulum; the mother's brother had isolated HSCR. In one family SS-HSCR occurred as an isolated malformation in the proband (A134) and in association with congenital deafness in the brother of this patient (A135). In one family, a girl with SS-HSCR (A18) had a brother with LS-HSCR (A17). Apart from SS-HSCR, the proband also had craniofacial dysmorphisms, syndactyly and brachydactyly, and was mentally retarded. Her brother, A17, had similar craniofacial dysmorphisms, while another brother shared the craniofacial dysmorphisms and the mental retardation but did not have HSCR.

The family history of 5 SS-HSCR patients without associated malformations revealed 10 family members with several congenital malformations other than HSCR. In the family of patient B1, cardiac septal and neural tube defects occurred. In the family of patient A79, three cases with (cheilo)(gnatho) palatoschisis were present. Pigment abnormalities were present in family members of patients B21 and A48.

Long-segment HSCR

We ascertained 44 LS-HSCR patients of whom 20 had associated malformations and/or dysmorphisms (45.5%). We ascertained one male LS-HSCR patient with Down syndrome. The length of the aganglionic segment in this patient was ca. 20 cm. Nine other 'syndromic' cases of LS-HSCR were identified (20.5%; ♂ to ♀, 1 to 1). This class (III) included one female patient (A42) with a mosaicism of an abnormal chromosome 11. The latter patient was characterized by psychomotor retardation, palatoschisis, webbed neck, hypoplastic nails and an ectopic anus. The 'syndromic' LS-HSCR patients had both major/minor malformations and craniofacial dysmorphisms (Table VI). Waardenburg syndrome type 2 was present in cases A25 and B5; Ondine's curse and neuroblastoma was present in patient B45. In the other 5 cases a syndrome diagnosis was not made. Class IV patients had associated craniofacial dysmorphisms (11.4%; ♂ to ♀, 1 to 1). Facial dysmorphisms included hypertelorism, epicanthus, low set and tilted ears, bifid earlobe, broad nasal bridge, small nose and mouth, short philtrum and micrognathia. Class V entails 5 patients with one other congenital malformation (11.4%; ♂ to ♀, 4 to 1). Three patients (B52, A56, and A23) had abnormalities of the urogenital tract (hypoplastic or aplastic right kidney), and another had an eventration of the left diaphragm. Patient A51 had a atrio-ventricular septal defect and tricuspid valve atresia.

Familial occurrence of LS-HSCR and/or associated anomalies is presented in Table VII. We identified two families with isolated LS-HSCR which presented 7 patients with aganglionosis (5♂; the sex of the other two cases was unknown). In one family two boys had LS-HSCR. In another family, LS-HSCR occurred in the proband while aganglionosis of

Table IV: Malformations and/or dysmorphisms associated with SS-HSCR

Class	Pat.	Sex	Head	CNS	Eyes	Ears	Mouth/nose	Heart	Other
III	B42	F+		Ondine's curse					Sacral dimple
III	B27	M			Narrow eyelids	Low set ear Eartag left	Tentmouth Micrognathia	VSD	
III	A3	F	Asymmetric face	Retardation Epilepsy	Hypertelorism				Pylorus hypertrophy
III	A152	M		Retardation Epilepsy		Bat ears	Broad nasal bridge		
III	B50	M	Sloping forehead	Nystagmus	Asymmetric eyelids Deep-set eyes	Large ears	Large nose Thin lips	Pulm. art. stenosis	Sacral dimple
III	B49	M				Tilted ears	Prominent philtrum and upper lip; Micrognathia		Cutaneous syndactyly Meckel's diverticulum
III	A18	F	Microcephaly		Blue sclera	Abnormal ears	Broad nasal bridge		Brachydactyly of big toes Syndactyly of dig. 2-3 both feet
III	A48	F				Congenital deafness			Adactyly of dig. 2-4 both feet
III	A24	F				Deafness			
III	A135	M				Deafness			

IV	A53	M	Epicanthal folds		Broad nasal bridge		
IV	B17	M	Antimongoloid eyes				
IV	B20	F		Dysplastic low set ears	Micrognathia		
IV	B58	M		Ear tag			
V	B35	M				Pulm. art. stenosis	
V	A114	M					Meckel's diverticulum
V	A78	M					Meckel's diverticulum
V	A102	M					Meckel's diverticulum
V	A75	M					Meckel's diverticulum
V	B12	M					Meckel's diverticulum; Sacral dimple
V	B40	M					Single umbilical artery
V	B53	M					Polydactyly; Rockerbottom feet
V	A72	F					Hydronephrosis; Hydro-ureters; reflux
V	B43	F					Hemihypertrophy R
V	A19	M					Vesico-ureteral reflux L
V	A33	M					Vesico-ureteral reflux L

Table V: Familial occurrence of SS-HSCR and/or associated anomalies

Pat.	Sex	Associated anomalies	Familial aganglionosis	Associated anomalies in family members
A89	M		Father	
A90	M		3 cases in family	
B16	M		Father, 2 sisters of father	
A164	M		Brother of A165	
A165	F		Sister of A164	
B4	F		Mother's father	
B50	M	Craniofacial dysmorphisms Nystagmus	Father's brother	
A114	M	Meckel's diverticulum	Mother's brother	
A134	M		Brother of A135	
A135	M	Deafness	Brother of A134	
A18	F	Retardation Syndactyly Craniofacial dysmorphisms	Sister of A17	Brother: dysmorphisms, retardation
B1	M			Sib: cardiac septum defect; Father's sister: cardiac valve anomaly; Mother's sister: Child with Down syndrome, and child with neural tube defect
B21	M			Father: white forelock
A79	M			Brother, father and brother of father: (cheilo)(gnatho)-palatoschisis
A150	M			Sib: spina bifida
A48	F			Mother: polychromatic iris

unknown length occurred in his father, 2 sibs of the father, and father's uncle. Associated malformations and/or dysmorphisms were not reported in these families. LS-HSCR was associated with other congenital malformations in 2 families. In one family Waardenburg syndrome type 2 was diagnosed (A25 and B5). In the other family, two sibs of the proband had Potter's syndrome.

The incidence of associated anomalies and sex ratios of the various classes of SS- and LS-HSCR are summarized in Table III. Statistical analysis revealed that 1) There is no

significant difference between SS-HSCR and LS-HSCR with regard to sex ratio ($p = 0.605$). 2) LS-HSCR is more frequently associated with anomalies than SS-HSCR ($p < 0.005$); 3) Down syndrome is most frequently associated with SS-HSCR ($p < 0.05$). 4) There are significant differences with regard to sex-ratios in class III and IV compared to class I ($p < 0.0001$).

Discussion

As an initial step to study possible pathogenetic mechanisms of one or more types of HSCR, we determined the incidence and nature of associated malformations and/or dysmorphisms in patients. Establishing the occurrence of associated malformations might help in syndrome delineation.

Retrospective analysis of 214 HSCR patients revealed an incidence of 27.6% of associated malformations and/or dysmorphisms. This percentage is much higher than that found in the normal population (2,3 to 5% of liveborn neonates [Méhes, 1988, Reerink et al., 1993]. The incidence of congenital malformations associated with HSCR reported in the last 15 years ranges from 5.3% to 29.8% [Suzuki et al., 1978, Ikeda and Goto, 1984, Spouge and Baird, 1985, Moore et al., 1991, Ryan et al., 1992]. In two recent surveys of 370 and 179 HSCR cases respectively, the incidence of associated malformations amounted to 16.5% and 22% [Moore et al., 1991, Ryan et al., 1992]. It is likely that associated malformations have been underreported in the past, both in our institute and in the literature. This becomes particularly evident when we compare the incidence of associated malformations in our A group, admitted between 1970 and 1985 (19.8%) and B group, admitted between 1985-1992 (48.3%). It seems that routine evaluation by a dysmorphologist is an important factor in the detection of associated anomalies. When we considered SS-HSCR and LS-HSCR separately the incidence of associated anomalies amounted to 22.9% and 45.5% respectively. Ikeda and Goto [1984] also reported that the incidence of associated malformations increased with the length of the aganglionic segment (SS-HSCR 10.2%; total colonic aganglionosis 15.2%). They did not investigate the nature of the associated malformations.

Pattern of malformations and dysmorphisms associated with HSCR

In the majority of SS-HSCR and LS-HSCR cases in this study, aganglionosis seems to be an isolated defect (77.1% and 54.5% respectively). In 22.9% and 45.5% of cases, SS- and LS-HSCR occurred in association with one or more congenital malformation. We classified these remaining cases depending on the phenotype of the associated malformations. We found that SS-HSCR and LS-HSCR do not differ markedly with respect to the pattern of associated anomalies, sex ratio, and familial occurrence. It might well be that isolated forms of short-

Table VI: Malformations and/or dysmorphisms associated with LS-HSCR

Class	Pat.	Sex	Head	CNS	Eyes	Ears	Mouth/nose	Heart	Other
III	A103	M	Flattened forehead	Retardation Epilepsy	Epicanthus Horner L	Low set ears	Large mouth Micrognathia		Hypospadia; Partial syndact. dig. 2-3 L hand
III	B11	M	Protruding forehead		Epicanthus L		Broad nasal bridge	Truncus arteriosus	Abnormal footcreases
III	A42	F		Retardation			Palatoschisis		Webbed neck; Ectopic anus (chrom 11 abn.)
III	B54	F+	Round head Microcephaly		Hypertelorism Iris coloboma	Dysplastic R ear Deafness	Micrognathia	Pulm. art. stenosis	Dysplastic kidneys
III	B30	M			Hypertelorism				Hypoplastic R kidney Widow's peak; Syndactyly
III	B33	M					Micrognathia	Pulm. art. stenosis	Syndactyly
III	A25	F			Pale irides	Dysplastic ears; Congenital deafness	Broad nasal bridge		
III	B45	F+		Ondine's curse					Neuroblastoma
III	B5	M+			Pale irides	Dysplastic ears			White hairlock; Sacral dimple; Lumbal patch of hair

IV	A17	M+	Short neck		Low set ears	Broad nasal bridge		
IV	B41	F				Micrognathia		
IV	B31	F	Round head Sloping forehead	Hypertelorism		Small nose Small mouth		
IV	A7	F			Bifid earlobe L			
IV	B32	M+		Epicanthus	Tilted ears	Short philtrum Micrognathia		Abnormal hand crease R
V	B52	M						Vesico-ureteral reflux R
V	A8	M						Eventration of diaphragm L
V	A56	M						Hypoplastic R kidney
V	A51	M+					AVSD; tricuspid atresia	
V	A23	F+						Abnormal handcreases Aplastic R kidney

and long segment disease are variant forms of the same genetic defect.

The association of HSCR and Down syndrome has since long been recognized [Wolf and Zweymueller, 1962, Emanuel et al., 1965]. The incidence of Down syndrome in our study (6.5%) falls within the 2% - 14% range reported in the literature [Passarge, 1967, Polley and Coran, 1986]. It is striking that 13 of the 14 cases had SS-HSCR. This is in accordance with the study by Badner and coworkers [1990]. In addition, the male to female preponderance of 6 : 1 in our series was also noticed by this group (10.5:1). We were not able to determine whether this male preponderance in the combination of SS-HSCR and Down syndrome also occurred in other large series since the sex of the Down cases is rarely mentioned. Trisomy 21 is a major cause of congenital malformations of the heart and digestive tract and of mental retardation. Recent work has suggested small regions in band q22 that are likely to contain genes for some of these features [Korenberg et al., 1992]. However, until now no linkage between this region and HSCR has been reported [Slaugenhaupt et al., 1991]. Various nonspecific mechanisms such as increased cell adhesion and limited cell proliferation, have been suggested as pathogenetic mechanisms for the malformations, but the relationship of these cellular events to chromosome 21 imbalance is not understood.

In total, 19 'syndromic' cases were recorded, representing 5.9% of the SS-HSCR cases and 20.5% of the LS-HSCR. Some of these malformations and dysmorphisms associated with HSCR are similar to known Mendelian disorders or partially resemble them. A syndrome diagnosis was obtained only in 5 cases (two Ondine's curse (OMIM 209880), three cases with Waardenburg syndrome type 2). The association of HSCR and Waardenburg syndrome type I is the best known [Omenn and McKusick, 1979, Badner and Chakravarti, 1990]. Waardenburg syndrome manifests as variable combinations of deafness and pigment abnormalities such as heterochromia iridis, white forelock and white skin patches. Clinically, Waardenburg syndrome is divided into two main subtypes which usually breed true within families and which have been supposed to be genetically distinct. The two types are distinguished by the presence in type 1 of dystopia canthorum, an outward displacement of the inner canthi of the eyes. Waardenburg type 1 has been attributed to mutations in the *PAX3* gene. There is no consensus whether mutations in *PAX3* can also lead to Waardenburg syndrome type 2. Until now no linkage between the *PAX3* region on chromosome 2 and HSCR has been reported [Slaugenhaupt et al., 1991]. Waardenburg syndrome type 2 was found to be associated with both SS-HSCR and LS-HSCR within one family (patients A25 and B5). We identified five families in which the proband suffered from isolated SS-HSCR while other family members had features occurring in the Waardenburg syndrome (white forelock, polychromatic iris, spina bifida). It might well be that the association of HSCR and Waardenburg syndrome type 2 is an autosomal dominant trait with variable penetrance and expression. Detailed family histories of sporadic HSCR cases might help to elucidate this issue.

Table VII: Familial occurrence of LS-HSCR and/or associated anomalies

Pat.	Sex	Associated anomalies	Familial aganglionosis	Associated anomalies in family
B24	M		Father, 2 sibs of father, father's uncle	
A20	M		Brother of A21	
A21	M		Brother of A20	
A17	M	Craniofacial dysmorphisms	Brother of A18	
A25	F	Waardenburg type 2	Sister of B5	
B5	M	Ear malformations Piebaldism Micrognathia	Brother of A25	
B32	M	Craniofacial and other dysmorphisms		Two sibs: Potter syndrome

A clear 'syndrome' diagnosis was not made for the sibs A18, A17. One brother had similar dysmorphisms but no HSCR. These patients resemble the cases described by Hurst et al. [1988]. HSCR patients without overt congenital malformations but with craniofacial dysmorphisms might also be included in the 'syndromic' cases of HSCR.

In 17 patients (7.9%), HSCR was associated with one other congenital malformation but without craniofacial dysmorphisms. We found Meckel's diverticulum in 3.8% of patients, compared to 2% in the general population [Turgeon and Barnett, 1990, St-Vil et al., 1991], and to 0.5% in another group of HSCR patients [Ikeda and Goto, 1986]. Cardiac defects and single umbilical artery have been observed as non-random occurring associated anomalies. These isolated congenital malformations can occur together in the VATER or VACTERL association. A co-occurrence of HSCR and a full blown VACTERL association has been reported by Ryan et al. [1992]. In other studies association of HSCR with either anal atresia, limb, cardiac, or urinary tract malformations have been reported [Spouge and Baird, 1985, Takada et al., 1985, Ikeda and Goto, 1986, Watanatittan et al., 1991].

We classified HSCR into at least four different classes based on the presence and nature of associated malformations. The developmental biology of the enteric nervous system might help in elucidating the pathogenesis of the various forms of HSCR. The neurons and

supportive cells of the vertebrate enteric nervous system derive from neural crest cells originating from the posterior hindbrain. During an early phase of development, the hindbrain is divided into eight repeating units or rhombomeres [Keynes and Lumsden, 1990, Lumsden et al., 1991, Noden, 1991]. In the anterior hindbrain, the neural crest is segregated into streams. It is unknown whether neural crest cells in the posterior hindbrain are also segregated into streams. Ablation of the neural crest adjacent to somites 3 through 5 resulted in aganglionic colon without any other obvious malformations. Ablation of the neural crest of rhombomeres 6 through 8 resulted in total intestinal aganglionosis, thymic aplasia or hypoplasia, and disturbances in the cardiac outflow tract [Kirby, 1993, Peters-van der Sanden et al., 1993]. Apparently, the longer the aganglionic segment the higher the incidence of associated malformations. Based on these experimental data one would predict that the malformations associated with HSCR reside in derivatives of the neural crest of the posterior hindbrain (or third and fourth pharyngeal arches). This holds true for the 7 cases with cardiac outflow defects in our series and cases with cardiac defects (e.g. tetralogy of Fallot) in other studies [Spouge and Baird, 1985, Ryan et al., 1992]. This might also hold true for abnormalities in the thyroid and parathyroids as these occur in the MEN-2A syndrome [Verdy et al., 1982]. However, these abnormalities consist of tumors instead of congenital malformations. An association with thymic hypo-or aplasia has not been reported.

Craniofacial dysmorphisms and malformations can be related to the cranial neural crest since this crest contributes to the ectomesenchymal structures in nasal, orbital, maxillary skeleton, palate, cranial bones, and the otic capsule, as well as to connective tissue of the face and ventral part of the neck [Couly et al., 1993].

Genetic factors and HSCR

Several lines of evidence suggest the involvement of genetic factors in the pathogenesis of HSCR i) the elevated risk to sibs; ii) the dominant pattern of inheritance in several families with LS-HSCR; iii) the association with trisomy 21, microdeletions of chromosome 13q, and 10q; iv) the presence of linkage of LS-HSCR with a locus on chromosome 10q11; and v) the existence of Mendelian models for colonic aganglionosis in rodents. The pattern of inheritance, however, does not appear to be due to a single gene in all families.

We identified 8 familial cases of isolated HSCR (4.2% of all cases). There was no apparent difference with regard to the incidence of familial cases between familial SS- and LS-HSCR cases. When 'syndromic' cases are included, however, familial occurrence increased with the length of the aganglionic part of the bowel (6.5% vs 14%; the overall incidence of familial cases is 7.9%). This has been observed previously [Passarge, 1967, Badner et al., 1990, Moore et al., 1991].

The majority of our HSCR patients were not karyotyped. There are only few examples in the recent literature concerning cytogenetic abnormalities in patients with intestinal

aganglionosis. Of particular interest is the observation by Martucciello et al. [1992] of a small cytogenetically visible deletion of chromosome 10q11.2-q21.2 in a female patient with LS-HSCR without any other detectable anomalies. This particular patient led to the mapping of familial LS-HSCR to a locus on the proximal long arm of chromosome 10 (10q11.2) between D10S208 and D10S196 [Angrist et al., 1993, Lyonnet et al., 1993]. Edery et al. (submitted for publication) described the c-*RET* proto-oncogene as the closest genetic marker with respect to the disease locus, suggesting that this proto-oncogene, which has been shown to account for multiple endocrine neoplasia type 2A, might be a candidate gene for HSCR. For both familial LS- and SS-HSCR, tight pairwise linkage with no recombination events was observed between c-*RET* and the disease loci (personal communication), suggesting that familial SS-HSCR and LS-HSCR are allelic disorders. It might well be that chromosome 10q11 contains a set of genes that are important for the development of the rhombencephalic neural crest.

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3.8. General discussion

Finally, I would like to try and make an inventory of the certainties and, perhaps even more importantly, the uncertainties, of ENS development. Because of its complexity, I subdivided this process into a number of separate components, although one should realize that such a subdivision is somewhat artificial.

1. Segmentation within the neural crest regarding enteric nervous system development
In recent years, research interest has become focused on segmentation of the neural crest, especially in the region of the rhombencephalon. It was shown that within this region, small neural crest segments gave rise to specific derivatives (Noden, 1983; Lumsden et al., 1991). In the posterior rhombencephalon, ectomesenchymal derivatives originate mainly from the cardiac neural crest from the level of the mid-otic vesicle down to the caudal boundary of somite 3 (rhombomeres 6-7) (Kirby et al., 1983; Bockman and Kirby, 1984). Inversion of this part of the neural crest resulted in defective cardiac development, further indicating its segmental character (Kirby, personal communication). A major finding in this thesis is, that the neural crest adjacent to somites 3-5 (rhombomere 8, and perhaps part of rhombomere 7) is specifically responsible for the innervation of the hindgut (colon) in vivo. Construction of isotopic and isochronic quail-chick chimeras showed that this small segment of the posterior rhombencephalic neural crest could even be responsible for the innervation of the entire digestive tract (LeDouarin and Teillet, 1973) (Peters-van der Sanden et al., unpublished results). We surmise that the posterior rhombencephalic neural crest is segmented with regard to the formation of the ENS. In vivo, the innervation of the hindgut, and possibly of the entire gut, specifically depends on the neural crest adjacent to somites 4-5.

The molecular basis for the segmentation of the rhombencephalic crest could be formed by the differential antero-posterior expression of members of the homeobox-containing gene family. In mice, study of the expression pattern of these genes in both the neuroepithelium and the paraxial mesoderm has shown that each axial segment expresses a specific combination of genes, providing it with a so-called *Hox* code (Hunt and Krumlauf, 1992). Analysis of transgenic mice bearing targeted germline mutations in members of the *Hox-A* gene cluster has provided direct experimental evidence that these *Hox* genes play an important role in hindbrain segmentation. A knockout mutation of the *Hox-A1* gene affected the development of cranial ganglia, whereas a knockout mutation of *Hox-A3* mainly affected ectomesenchymal derivatives of the rhombencephalic neural crest (Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992). Overexpression of the *Hox-A7* gene resulted in defects in the cervical vertebrae and in craniofacial abnormalities (Balling et al., 1989; Kessel et al., 1990), whereas overexpression of the *Hox-A4* gene resulted in disturbed ENS

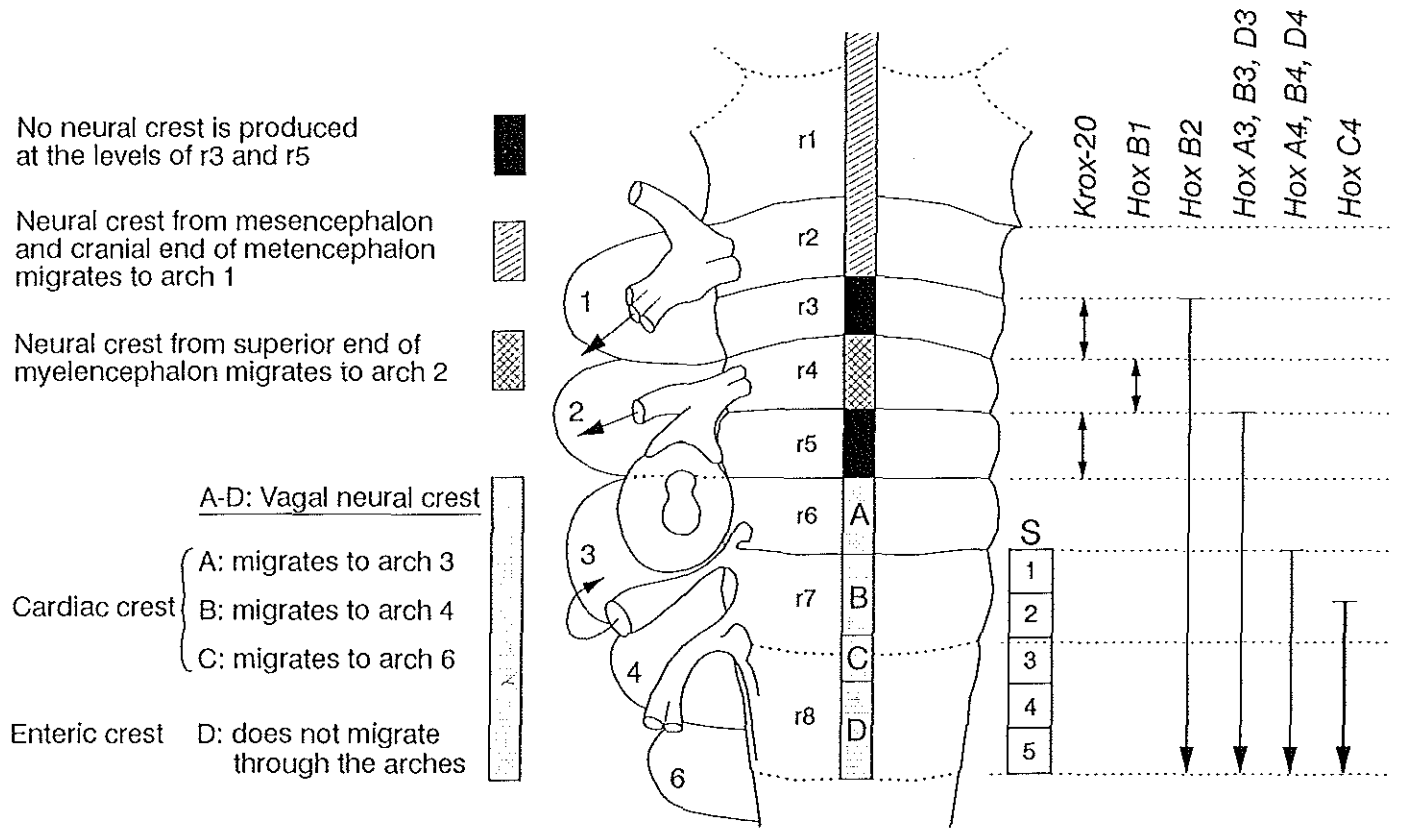

Figure 11: Schematic presentation of the various regions of the neural crest and their relation to the rhombomeres and pharyngeal arches. Segmentation within the neural crest is correlated with Hox gene expression in the neuroepithelium.

development (Wolgemuth et al., 1989). The fact that *Hox* gene expression can be altered by RA (Marshall et al., 1992), could provide the molecular mechanism that underlies the selective developmental defects seen in RA embryopathy (chapter 3.3).

In Fig. 11, segmentation of the posterior rhombencephalic crest with regard to the formation of its various derivatives, is correlated with the expression patterns of various *Hox* genes in mouse embryos. We surmise that the posterior rhombencephalic neural crest can be subdivided into the cardiac crest, from the midotic vesicle down to the caudal boundary of somite 3, and the enteric crest adjacent to somites 4-5. Detailed analysis of the expression patterns of the members of the various paralogous groups in both chicken and mouse embryos could provide further insight into the extent of segmentation of the rhombencephalic neural crest.

2. Migration of neural crest cells to the gut

In order to be able to contribute to ENS development, neural crest cells have to leave the neural tube, migrate to the gut and enter it at some point. There are still a number of largely unanswered questions, regarding these processes. First of all, when do neural crest cells, especially those at the level of somites 4-5, leave the neural tube? Studies using quail-chick chimeras have shown that most precursors for enteric neurons have left the neural folds prior to the 13-somite stage (HH stage 11) (LeDouarin and Teillet, 1973). Newgreen and Erickson (1986) showed that neural crest cell migration at this level occurred between somite stages 10-22 (HH stage 10-14). We showed that neural anlagen, consisting of neural tube, notochord and small somitic remnants, obtained from embryos having more than 18 somites (HH stage 13) were better capable of forming enteric ganglia than neural anlagen obtained from younger embryos (chapter 3.2.). These results might indicate that the enteric precursors either leave the neural crest later than hitherto assumed, or that they remain in close contact with the neural tube for a prolonged period of time. This delayed migration could represent some kind of induction or maturation process, dependent on close contact with the neural tube or notochord, necessary for proper ENS formation. Signals emanating from the neural tube were found to be necessary for the development of dorsal root ganglia in the trunk region (Kalcheim and LeDouarin, 1986; Kalcheim et al., 1987). Differentiation into enteric neurons, however, can occur in the absence of the notochord (Teillet et al., 1978).

Our second question concerns the migration pathways taken by the enteric neural crest precursors on their way to the gut. Recently, the migration pathways of rhombencephalic neural crest cells have been studied in great detail (Kuratani and Kirby, 1991; Lumsden et al., 1991; Miyagawa-Tomita et al., 1991; Sechrist et al., 1993). These studies have shown that cells from the rhombencephalic neural crest down to the caudal boundary of somite 3 migrate predominantly via a dorsolateral pathway on their way to the pharyngeal arches, whereas

caudal to somite 3, neural crest cells no longer enter the pharyngeal arches and instead migrate along a ventrolateral pathway through the anterior part of the somites. In this thesis, we show that the neural crest caudal to somite 3, is primarily responsible for ENS formation at least in the colon, indicating that ENS precursors might migrate along a ventrolateral pathway on their way to the gut. In vitro clonal analysis of the posterior rhombencephalic neural crest showed that the potential to differentiate into serotonergic neurons, which may constitute precursors for enteric neurons, decreased considerably upon entry into the pharyngeal arches (Ito and Sieber-Blum, 1993). Preliminary results, using microinjection of the lyophilic dye DiI, labelling a specific segment of the neural crest, further support the hypothesis that precursors for enteric neurons do not migrate through the pharyngeal arches (Raams et al., unpublished results). Previous studies that suggested that the enteric precursors migrate along a dorsolateral pathway, through the caudal pharyngeal arches (Thiery et al., 1982; Ciment and Weston, 1983; Payette et al., 1984; Tucker et al., 1986), were primarily based on indirect evidence provided by immunohistochemical localization of presumptive enteric precursors in tissue sections, using either an anti-neurofilament or the HNK-1 (NC1) antibody (Thiery et al., 1982; Payette et al., 1984; Tucker et al., 1986). These results provide only a static picture and do not show that cells, immunoreactive for a certain antibody, observed within the branchial arches, actually migrate to the gut. Additional evidence for a difference in migration pathways between anterior and posterior vagal neural crest cells comes from experiments, in which small segments of the vagal neural crest were cocultured with aneural hindgut on the chorioallantoic membrane. These experiments showed that all segments of the posterior rhombencephalic neural crest had the developmental potential to colonize the hindgut, indicating that there are no real intrinsic differences between these segments, and that the specific need for the neural crest adjacent to somites 3-5 for hindgut innervation might be due to a difference in migration pathways.

Using chorioallantoic membrane cocultures of chicken aneural and quail neural hindgut, we found that aneural gut was able to attract vagal neural crest cells which had already colonized a gut segment, provided ganglion formation in the neural gut was not yet fully completed (chapter 3.4.). Such a specific interaction between aneural hindgut and vagal neural crest cells was also found in heterotopic quail-chick chimeras in which the vagal neural crest was transplanted to the adrenomedullary region (LeDouarin and Teillet, 1974). In these chimeras, quail cells were found in all normal trunk derivatives, but in addition to these sites, they were also present in the postumbilical gut, a site which normally is not colonized by the adrenomedullary neural crest. These results indicate that vagal neural crest cells are specifically attracted by aneural gut, probably by signals emanating from the enteric mesenchyme.

It is generally agreed upon, that neural crest cells enter the pharynx at the third day of embryonic development (HH stage 17-18) (LeDouarin and Teillet, 1973; Tucker et al.,

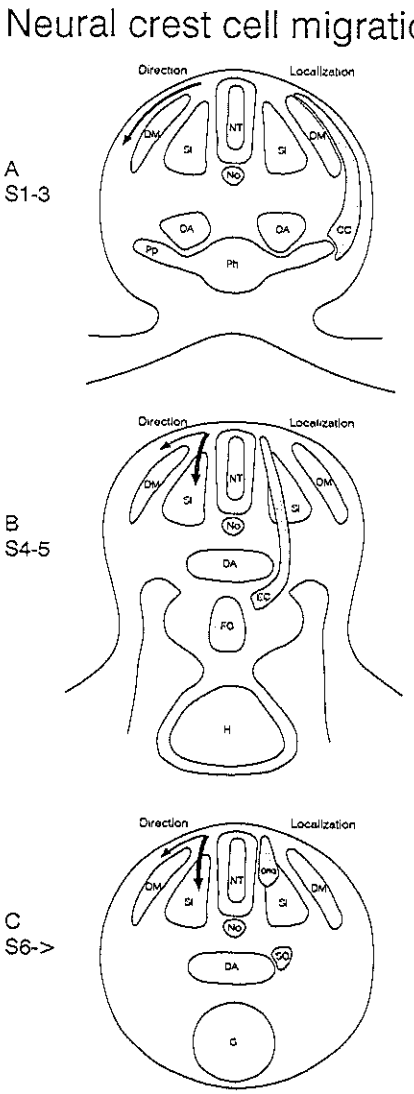

Figure 12: *Neural crest cell migration pathways at various levels in the vagal neural crest region. In the lefthand part of each picture, the relative importance of the dorsolateral (between ectoderm and dermamyotome) and ventral (through the anterior part of the somites) pathways is indicated by the relative size of the arrows. In the righthand picture the localization of the neural crest cells is indicated by the hatched areas. A) at the level of somites 1-3 B) at the level of somites 4-5 C) caudal to somite 6. NT= neural tube; No= notochord; DA= dorsal aorta; DM= dermamyotome; Sl= sclerotome; Ph= pharynx; Pp= pharyngeal pouch; CC= circumpharyngeal crest; Fg= foregut; H= heart; EC= enteric crest; G= gut; DRG= dorsal root ganglion; SG= sympathetic ganglion.*

1986), but the exact site of entry is hitherto unknown. Immunohistochemical data, using either the HNK-1 (Raams et al., unpublished results) or the E/C8 (Tucker et al., 1986) antibody, suggest that the point of entry lies at the level of the fifth or sixth somite, but definite proof should come from in vivo labelling studies using DiI or retroviral markers.

We surmise that the precursors for enteric neurons, predominantly arising at the level of somites 4-5, do not enter the pharyngeal arches, but migrate ventrolaterally through the anterior part of the somites and enter the pharynx at the level of somite 5 or 6 (Fig. 12).

3. Migration of neural crest cells through the gut

Once neural crest cells have entered the gut, they have to move caudally in order to give rise to enteric ganglia along the entire digestive tract. Whether this is achieved by active migration or by passive displacement along with the elongation of the gut, is still unclear. There are, however, some indications that, at least in the hindgut, active migration is taking place (Tucker et al., 1986). Study of the time course of neural crest migration through the gut showed that they colonize the foregut at E4. Subsequently, they migrate through the midgut at E5, reaching the level of the ceca approximately at E6.5. Migration is completed before E8 (LeDouarin and Teillet, 1973; Allan and Newgreen, 1980). Sacral neural crest cells have already colonized Remak's ganglion at E4, but they do not enter the hindgut prior to E7 (LeDouarin and Teillet, 1973; Pomeranz and Gershon, 1990; Pomeranz et al., 1991a; Serbedzija et al., 1991).

At the time neural crest cells move through the fore- and midgut the various layers of the gut wall have not yet formed, and the gut consists only of an endodermal tube surrounded by splanchnic mesoderm. By the time neural crest cells reach the hindgut the smooth muscle layers have formed, and neural crest cells are found on either side of these (LeDouarin and Teillet, 1973; Tucker et al., 1986). We found that neural crest cells initially migrate superficially under the splanchnic epithelium, forming the myenteric plexuses. Subsequently, they transverse the muscle layers to give rise to the submucous plexuses (Souren et al., unpublished results). In the fore-, and midgut, neural crest cells were found not to migrate as single cells, but by extending processes to their neighbours, thus form a complex network in the wall of the gut (Epstein et al., 1991). We found a similar network in the neural hindgut after wholemount staining using the HNK-1 antibody (Nurmohamed et al., unpublished results). Formation of such a network was found to be dependent on the presence of vagal neural crest cells and thought to be mediated by the enteric microenvironment.

4. Homing of neural crest cells in the gut

While migrating through the gut, neural crest cells have to receive a signal from the enteric

microenvironment, which tells them where to stop migration and form enteric ganglia. Upon study of the microenvironment of the aneural hindgut, we identified a layer of mesenchymal cells on the luminal side of the smooth muscle layer and cells at the serosal side, which reacted with the HNK-1 antibody (HNK-1 mode 1; chapter 3.5. and 3.6.). The localization of these HNK-1 immunoreactive cells exactly at the sites where ganglion formation occurs, suggests that these cells may present a homing signal to neural crest cells. In neural gut, these HNK-1 immunoreactive mesenchymal cells were no longer present and instead the HNK-1 antibody identified the enteric ganglia (HNK-1 mode 2). Using an adhesion assay, in which we incubated isolated neural crest cells on cryosections of aneural hindgut, we confirmed that these HNK-1 immunoreactive cells provided a homing signal for neural crest cells (unpublished results). We identified two HNK-1 carrying, cell membrane glycoproteins of 42 and 44 kD, but purification of these glycoproteins in sufficient amounts for further characterization and sequencing proved to be difficult, because of the limited amount of starting material (T. Luider, personal communication). Maybe the recently introduced technique of differential display using degenerate primers and PCR could provide a better tool (Liang and Pardee, 1992).

Pomeranz et al. (1991b) also provided evidence for an important role of the enteric microenvironment in the homing of neural crest cells. They found that neural crest cells acquire a receptor for laminin, while migrating through the gut. Laminin is an extracellular matrix molecule, which is normally present in the basal laminae of the mucosal and serosal epithelium and of the smooth muscle cells of the gut. The interaction between laminin and its receptor might cause neural crest cells to stop migrating and aggregate into enteric ganglia. Overabundance of laminin in a broad zone of the enteric mesenchyme, both in a mutant mouse strain with congenital megacolon (Payette et al., 1988) and in patients with Hirschspung disease (Parikh et al., 1992) also points to a possible role for laminin in ENS formation. In vitro analysis of cell-matrix interactions showed that the attachment of neural crest cells to extracellular matrix molecules is mediated predominantly by integrin receptors (Lallier and Bronner-Fraser, 1991). Furthermore, it was shown that cranial and trunk neural crest cells use a functionally distinct set of integrins to attach to different conformations of laminin (Lallier et al., 1992).

5. Differentiation of neural crest cells and the role of the enteric microenvironment

Using coculture experiments, we showed that the switch from HNK-1 mode 1 into HNK-1 mode 2 only occurred when neural crest cell colonization was followed by differentiation into enteric neurons (chapter 3.5.). When trunk neural crest cells colonized the hindgut, they differentiated mainly into melanocytes and the HNK-1 mode 1 immunoreactivity persisted. This indicates that the HNK-1 immunoreactive mesenchymal cells in aneural hindgut not only

present a migration and homing signal, but might also constitute a differentiation signal for neural crest cells. But, whereas the migration and homing signal can be recognized by all neural crest cells entering the hindgut, the differentiation signal is specific for vagal neural crest cells. Isolated vagal neural crest cells, which were cultured for one day in vitro, retained the specific characteristics enabling them to differentiate into enteric neurons in the hindgut. These characteristics, however, were lost upon prolonged in vitro culture, leading them to behave as trunk neural crest cells, differentiating into melanocytes.

Aberrant differentiation of trunk neural crest cells, which we observed in our coculture system, was also observed in a more in vivo situation. Le Douarin and Teillet (1974) constructed heterotopic quail-chick chimeras in which adrenomedullary neural crest was transplanted to the vagal region, and found that these quail trunk neural crest cells differentiated into melanocytes in the postumbilical gut. In the preumbilical gut, however, trunk neural crest cells were able to differentiate into enteric neurons. These results indicate that there may be intrinsic differences between the microenvironment of the various segments of the gut and that embryonic cell types respond differentially and specifically to these signals. Experimental evidence for such a segmentation within the digestive tract comes from our ablation experiments, in which we showed that there are differences between the various parts of the digestive tract regarding their dependence on specific neural crest segments (chapter 3.2.). Whereas the hindgut depended specifically on the neural crest of somites 3-5, the midgut could be colonized by the entire posterior rhombencephalic neural crest. The foregut could also be innervated by a source outside the vagal neural crest, presumably by more anterior rhombencephalic neural crest. Transgenic mice with a null mutation of the *ret-1* gene had total intestinal aganglionosis, but they, too, had a normally innervated foregut (V. Pachnis, personal communication).

These experimental data strongly suggest that there are qualitative differences in the microenvironment of the various parts of the digestive tract. A quantitative effect, however, caused merely by the amount of vagal neural crest cells that eventually reaches the gut, can not be excluded. Such a quantitative effect, however, would be expected to result in a gradual transition between the ganglionic and aganglionic parts of the gut. We, however, found sharp boundaries localized at morphologically identifiable sites, one at the level of the foregut-midgut transition, which is the site were the pancreas develops, and the other at the ileo-cecal junction. The fact, however, that both the pancreas and the ceca are colonized by neural crest cells, might suggest that the migrating neural crest cell-network arrests temporarily at these sites until either the pancreas or the ceca are colonized, thereby increasing the chance that the transition of ganglionic to aganglionic gut is found at these sites.

We conclude that neural crest cells carry receptors that recognize signals in the enteric microenvironment, which enable them to migrate through the gut, home at specific sites and differentiate into enteric neurons. The microenvironment of the postumbilical gut harbors

signals, that differ from those in the preumbilical gut, and only vagal neural crest cells are able to recognize these specific differentiation signals presented by the HNK-1 immunoreactive cells. Trunk neural crest cells recognize the migration and homing signals, but lack the specific receptor necessary for correct differentiation and instead differentiate into melanocytes. In order to further characterize these signals, the effect of various known differentiation factors could be tested on in vitro cultured precursors for enteric neurons.

6. Clinical implications

A deletion of the long arm of chromosome 10 (10q11) has been found in a patient with HSCR (Martucciello et al., 1992). Recently, linkage has been established between HSCR and the *c-RET* proto-oncogene, which maps to this region (Edery et al., submitted). The finding that transgenic mice carrying a null mutation of the *ret-1* gene have total intestinal aganglionosis, further suggests that the *RET-1* gene might be involved in HSCR (Dr. V. Pachnis, personal communication). Members of families in which linkage with *c-RET* was established, suffered from isolated aganglionosis with no associated anomalies. In our clinical study (chapter 3.7.), however, we found that HSCR could also be associated with other major/minor congenital malformations and/or dysmorphisms. HSCR patients were classified into five groups based on the spectrum of these associated anomalies, both in sporadic and familial cases of HSCR. This could indicate that multiple pathogenetic mechanisms are involved in HSCR and stresses the importance of precise syndrome delineation and recording of aganglionosis and/or other anomalies in family members, in large series of prospective case-control studies.

Search for chromosomal abnormalities using high resolution banding techniques, and linkage analysis analysis in large pedigrees might lead to the identification of other candidate regions which might harbor genes involved in HSCR. Chromosomal aberrations already found to be associated with HSCR are trisomy 21 (associated with Down syndrome), a deletion or trisomy of 22q11 (Passarge, 1967; Spouge and Baird, 1985; Beedgen et al., 1986), and microdeletions of the long arm of chromosome 13 (in the regionn 13q22-33) (Sparkes et al., 1984; Kiss and Osztovics, 1989; Lamont et al., 1989; Bottani et al., 1991). Recently, certain modifyer genes, influencing the phenotypic expression of the disease and its associated malformations, have been identified for Neurofibromatosis type I (Easton et al., 1993). To discriminate between the possibilities of multiple genes for HSCR or one primary gene, phenotypically influenced by modifyer genes, further research is necessary.

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SUMMARY

In this thesis, we studied the development of the ENS and the specific role of the hindbrain neural crest in this process, in avian embryos. The vertebrate neural crest arises on the dorsal aspect of the neural tube along the entire antero-posterior axis of the embryo. Cells from the neural crest migrate throughout the body, and give rise to a large variety of derivatives. The hindbrain or rhombencephalic neural crest gives rise to the intrinsic component of the ENS, a complex, integrative system of neurons and supportive cells embedded in the wall of the gut. The ENS regulates bowel motility and in many aspects resembles the central nervous system. In chapters 1 and 2, we introduce the neural crest and the enteric nervous system. We summarize the current knowledge on these structures and compare their embryonic development in various animal species. This showed that although there are some species specific variations, the basic principles are very similar.

The rhombencephalon consists of eight consecutive rhombomeres. This segmentation is also found in its neural crest. Cells derived from the first five rhombomeres migrate in two separate streams and populate the first two pharyngeal arches. Segment identity is established through the expression of a specific combination of *hox* genes within each rhombomere and its neural crest, providing it with a so-called *hox* code. Neural crest cells retain this code during migration.

The cellular and molecular mechanisms of the development of the neural crest of rhombomeres 6-8 are less well known. In chapter 3, we studied whether the neural crest is regionally specified with regard to ENS formation. We showed that the enteric ganglia of the hindgut, and perhaps even of the entire gut, derive from the neural crest adjacent to somites 4-5. Ectomesenchymal derivatives, such as cells of the outflow tract of the heart, and the mesenchymal component of thymus and parathyroids, derive from the neural crest adjacent to somites 1-3. Cells from rhombomeres 6-8 migrate either dorsolaterally, entering the third, fourth and sixth pharyngeal arches, or ventrolaterally through the rostral part of the first five somites. Neural crest cells in the pharyngeal arches mainly give rise to ectodermal derivatives. Clonal analysis showed that most pluripotent neural crest cells loose the capacity to differentiate into serotonergic neurons, upon entry into the pharyngeal arches. We surmise that ENS precursors migrate along a ventrolateral pathway and do not enter the pharyngeal arches, in contrast to other rhombencephalic neural crest cells.

Upon entry into the foregut, cells translocate craniocaudally, either as dispersed cells or in a network, home at the sites of the myenteric and submucous plexuses and differentiate into enteric neurons. We studied which cells or tissues within the gut wall provide homing and/or differentiation signals for neural crest cells. We identified a group of mesenchymal cells, carrying two HNK-1 immunoreactive cell membrane glycoproteins of 42 and 44 kD.

These cells were only found in those parts of the gut which were not yet colonized by neural crest cells, or in which neural crest cells did not differentiate into neurons. We surmise that these cells present signals, recognized by neural crest cells, enabling them to home at specific sites and differentiate into enteric neurons. Differentiation signals in the postumbilical gut, however, differ from those in the preumbilical gut, in that only vagal neural crest cells are able to recognize these. Trunk neural crest cells recognize the migration and homing signals, but lack specific receptors necessary for correct differentiation and instead differentiate into melanocytes.

Congenital malformations of the enteric nervous system in humans give rise to significant morbidity and even mortality in early childhood. The most common form is Hirschsprung disease, in which neurons are lacking in the distal colon (aganglionosis). Study of Hirschsprung disease could provide insight into the development of the enteric nervous system. A prerequisite for a molecular genetic approach of congenital malformations requires a precise characterization of patient groups. In a retrospective study of patients with Hirschsprung disease, we found a pattern of associated anomalies. We showed that there is no real difference between short- and long-segment disease regarding this pattern of associated anomalies, the sex-ratios and familial occurrence. A number of chromosomal abnormalities have been identified in Hirschsprung patients, indicating that there could be multiple genes involved in causing aganglionosis. Recently, linkage has been established between Hirschsprung disease and the *c-RET* proto-oncogene on the long arm of chromosome 10 (10q11). Future attention will be focused on the role of this gene or genes in its immediate vicinity in various groups of Hirschsprung patients.

SAMENVATTING

In dit proefschrift hebben wij de ontwikkeling van het zenuwstelsel van de darm en de specifieke rol van de rhombencephale neuraallijst hierin bestudeerd in vogel embryonen. De neuraallijst in vertebraten ontstaat aan de dorsale zijde van neuraalbuis over de gehele antero-posterior as van het embryo. Cellen afkomstig van deze neuraallijst migreren door het embryo, en geven aanleiding tot een grote verscheidenheid aan derivaten. De neuraallijst cellen van de achterhersenen of rhombencephalon vormen de intrinsieke component van darminnervatie, een complex, geïntegreerd systeem van neuronen en steuncellen in de darmwand. Het zenuwstelsel van de darm reguleert de darmmotiliteit en lijkt in vele opzichten op het centrale zenuwstelsel. In de hoofdstukken 1 en 2, worden de neuraallijst en het zenuwstelsel van de darm geïntroduceerd. We geven een samenvatting van de huidige kennis over deze structuren en vergelijken hun embryonale ontwikkeling in een aantal diersoorten. Deze vergelijking geeft aan dat, hoewel er enkele diersoort-specifieke variaties zijn, de basale principes sterk overeenkomen.

Het rhombencephalon bestaat uit acht opeenvolgende segmenten of rhombomeren. Deze segmentatie wordt teruggevonden in de bijbehorende neuraallijst. Cellen afkomstig van de eerst 5 rhombomeren migreren in twee afzonderlijke stromen en bevolken de eerste twee kieuwbogen. De identiteit van de afzonderlijke rhombomeren en de bijbehorende neuraallijst wordt bepaald door de expressie van een specifieke combinatie van *hox* genen, resulterend in een zogenaamde *hox* code. Neuraallijst cellen behouden deze code gedurende hun migratie.

De cellulaire en moleculaire mechanismen van de ontwikkeling van de neuraallijst van de de rhombomeren 6-8 zijn minder bekend. In hoofdstuk 3 wordt aannemelijk gemaakt dat de neuraallijst van rhombomeer 6-8 regionaal gespecificeerd is met betrekking tot de ontwikkeling van de darminnervatie. Wij hebben aangetoond dat de darmwand neuronen in de einddarm, en mogelijk zelfs in de gehele darm, afkomstig zijn van de neuraallijst ter hoogte van de somieten 4 en 5. Ectomesenchymale derivaten, zoals het uitstroom septum van het hart en de mesenchymale component van de thymus en bijschildklieren, daarentegen, zijn voornamelijk afkomstig van de neuraallijst ter hoogte van de eerste 3 somieten. Cellen afkomstig van de rhombomeren 6-8 migreren ofwel dorsolateraal, naar de derde, vierde en zesde kieuwboog, ofwel ventrolateraal door het rostrale deel van de eerst vijf somieten. Neuraallijst cellen in de kieuwbogen geven nagenoeg uitsluitend aanleiding tot ectomesenchymale derivaten. Clonale analyse heeft aangetoond dat de meeste pluripotente neuraallijst cellen het vermogen verliezen om te differentiëren in serotonerge neuronen, na binnenkomst in de kieuwbogen. Wij veronderstellen dat de precursors voor de darmwand neuronen langs ventrolaterale weg migreren en niet via de kieuwbogen, in tegenstelling tot andere rhombencephale neuraallijst cellen.

Na binnenkomst in de voordarm, verplaatsen neuraallijst cellen zich craniocaudaal, hetzij afzonderlijk, hetzij in een netwerk, en, na het bereiken van hun bestemming (de plaats van de plexussen van Auerbach en Meissner), differentiëren tot darmwand neuronen. Wij hebben onderzocht welke cellen of weefsels in de darmwand 'homing' en/of differentiatie signalen leveren voor neuraallijst cellen. Wij hebben een groep mesenchymale in de darmwand geïdentificeerd, die twee HNK-1 immunoreactive celmembraan glycoproteïnen van 42 en 44 kD tot expressie brengen. Deze cellen kunnen uitsluitend worden aangetoond in darmdelen die nog niet door neuraallijst cellen zijn gekoloniseerd, of waarin neuraallijst cellen niet neuronaal zijn gedifferentiëerd. Wij veronderstellen dat deze cellen signalen presenteren, die herkent worden door neuraallijst cellen en hen in staat stellen hun specifieke bestemming te bereiken en te differentiëren tot darmwand neuronen. Differentiatie signalen in de post-umbilicale darm verschillen van die in de pre-umbilicale darm en alleen vagale neuraallijst cellen zijn in staat deze te herkennen. Romp neuraallijst cellen herkennen de migratie en 'homing' signalen, maar missen de specifieke receptoren nodig voor correcte differentiatie en in plaats daarvan differentiëren zij tot melanocyten.

Aanlegstoornissen van de darminnervatie bij de mens geven aanleiding tot ernstige morbiditeit en zelfs mortaliteit op de kinderleeftijd. De meest voorkomende vorm is de ziekte van Hirschsprung, waarbij darmwand neuronen ontbreken in het distale colon (aganglionosis). Bestudering van de ziekte van Hirschsprung kan inzicht verschaffen in de ontwikkeling van de darminnervatie. Een voorwaarde voor een moleculair genetische benadering van aangeboren afwijkingen vraagt een precieze karakterisering van patienten groepen. In een retrospectieve studie bij patienten met de ziekte van Hirschsprung, vonden wij een patroon in de geassocieerde afwijkingen. We hebben aangetoond dat er geen wezenlijk verschil is tussen kort- en lang-segment aganglionosis wat betreft dit patroon van geassociëerde afwijkingen, de geslachtsverhoudingen of het familiaal voorkomen. Een aantal chromosomale afwijkingen zijn geïdentificeerd in patienten met de ziekte van Hirschsprung. Dit suggereert dat er meerdere genen betrokken zouden kunnen zijn bij het ontstaan van aganglionosis. Recent is er koppeling aangetoond tussen familiale gevallen van de ziekte van Hirschsprung en het *c-RET* proto-oncogen gelegen op de lange arm van chromosoom 10 (10q11). Studie naar de rol van dit gen of nabij gelegen genen in verschillende groepen patienten met de ziekte van Hirschsprung en in proefdieren vormt een aandachtsveld voor toekomstig onderzoek.

Curriculum Vitae

Personalia:

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Opleiding en diploma's:

1981	Eindexamen Gymnasium ß, Augustinianum, Eindhoven
1984	Kandidaatsexamen Medische Biologie (B5*), Rijksuniversiteit Utrecht
1986	Proefdierkundige (ex. art. 9 Wet op de dierproeven)
1987	Stralingsdeskundige (4B)
1987	Doctoraalexamen Medische Biologie Hoofdvakken: - Identificatie van de verschillende subpopulaties cellen in het mammacarcinoom van de kat zowel in vivo als in vitro, m.b.v. polyclonale en monoclonale antilichamen, bij de vakgroep Veterinaire Pathologie, begeleiding Prof. Dr. E. Gruys. - Functionele studie naar de toxiciteit van deoxyadenosine voor adenosine-deaminase defiënte B lymfocyten, tijdens activatie en in rust, bij de vakgroep Immunologie, Wilhelmina Kinderziekenhuis Utrecht, begeleiding Dr. B.M. Zeegers. Bijvak: Algemene Dierkunde, projectgroep experimentele embryologie - Prof. Dr. W.L.M. Geilenkirchen
1988	Cambridge Proficiency Certificate, grade A
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Posities:

1987-1988	Tijdelijke aanstelling in het kader van een pilot-studie naar het effect van stress op het immuunsysteem, bij de vakgroep Immunologie van het Academisch Ziekenhuis Utrecht, Prof. Dr. R. Ballieux.
1988-1993	Thesis project getiteld: 'The hindbrain neural crest and the development of the enteric nervous system.' Dit onderzoek maakt deel uit van het Medisch Genetisch Centrum Zuid West Nederland (sectie 2D) en is bewerkt binnen een samenwerkingsverband van het Instituut

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List of publications

Peters-van der Sanden, M.J.H., Luider, T.M., van der Kamp, A.W.M., Tibboel, D., and Meijers, C. (1993) Regional differences between various axial segments of the avian neural crest regarding the formation of enteric ganglia. Differentiation 53:17-24.

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