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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

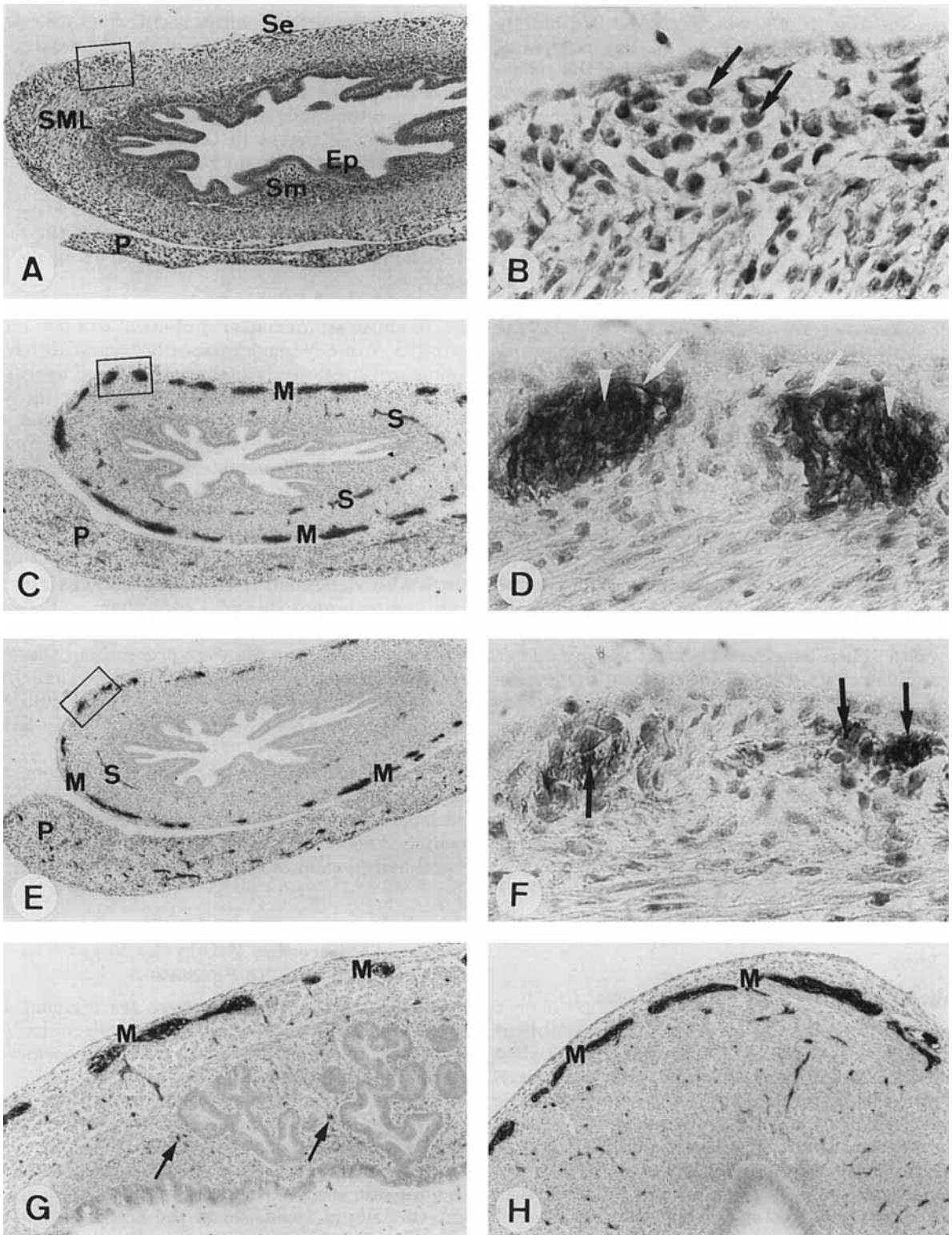

Fig. 1. Paraffin sections of E11 chicken gut after ablation the vagal neural crest from MO-S7 at stage 10, A–F: Duodenum. **A:** Hematoxylin staining showing the different layers in the duodenum. Ep: epithelium, Sm: submucosa, SML: smooth muscle layer, Se: serosa. P = pancreas. ×16. **B:** Detail of 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

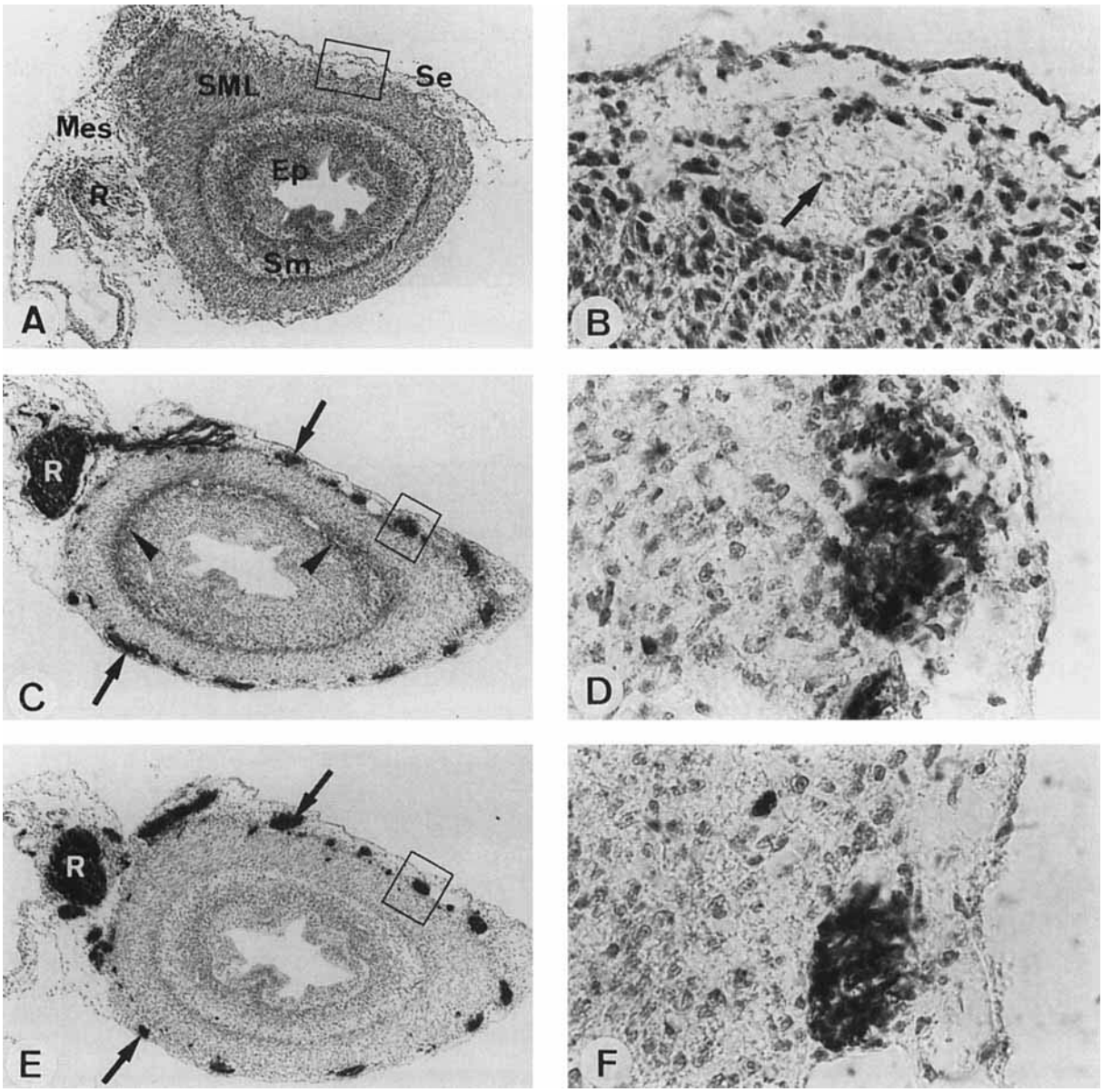

Fig. 2. Paraffin sections of E11 chicken colon after ablation of the vagal neural crest at the level MO-S7 at stage 10. **A:** hematoxylin staining showing the different layers in the colon. Ep: epithelium, Sm: submucosa, SML: smooth muscle layer, Se: serosa, Mes: mesentery, R: ganglion of Remak. × 16. **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-

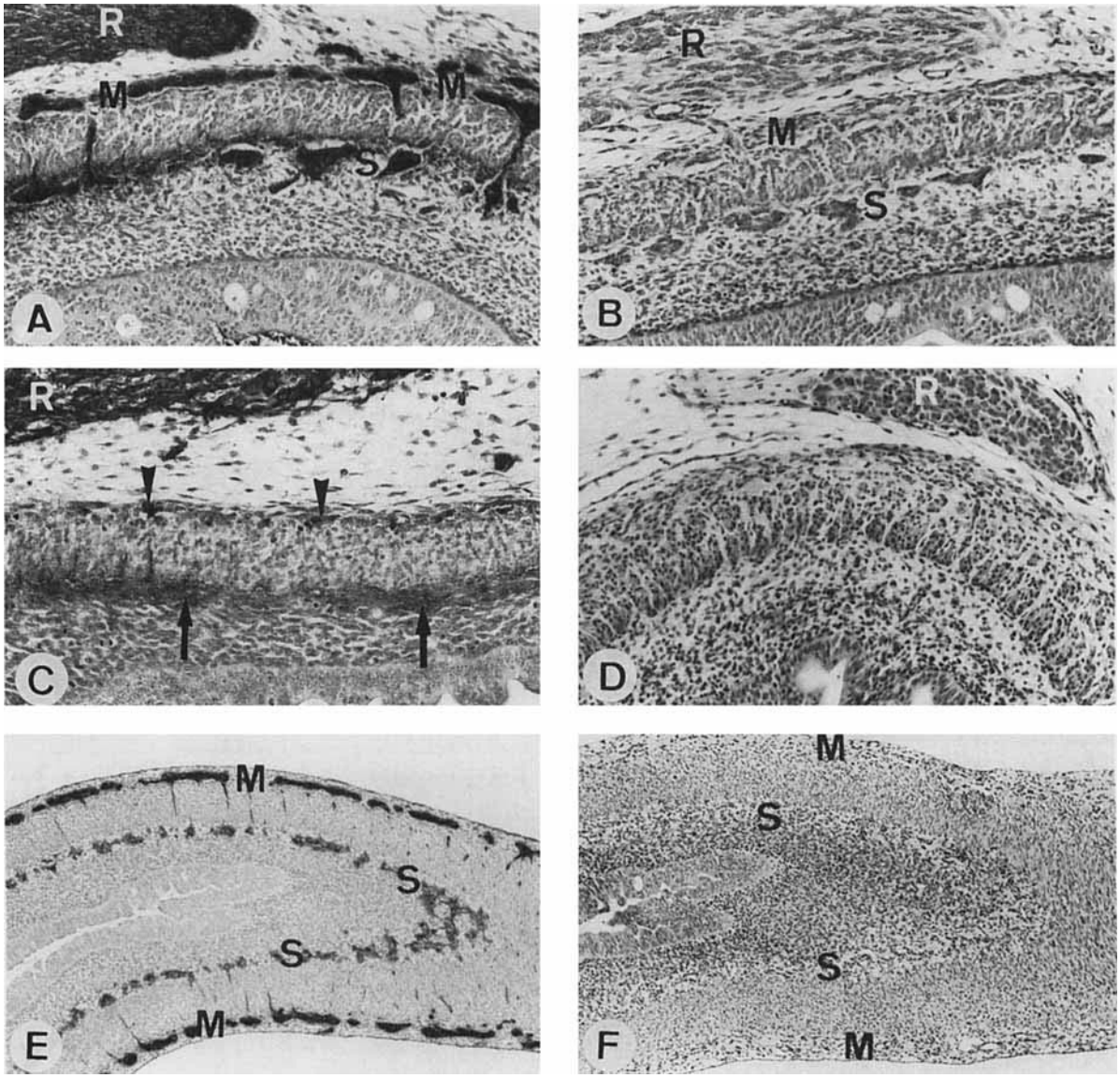

Fig. 3. Paraffin sections of E12 colon after ablation of part of the vagal neural crest at stage 10. **A:** Ablation at the level of the otic vesicle down to the caudal boundary of somite 3: HNK-1 immunoperoxidase staining showing the myenteric (M) and submucous (S) ganglia. Remak's ganglion (R) is also 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-

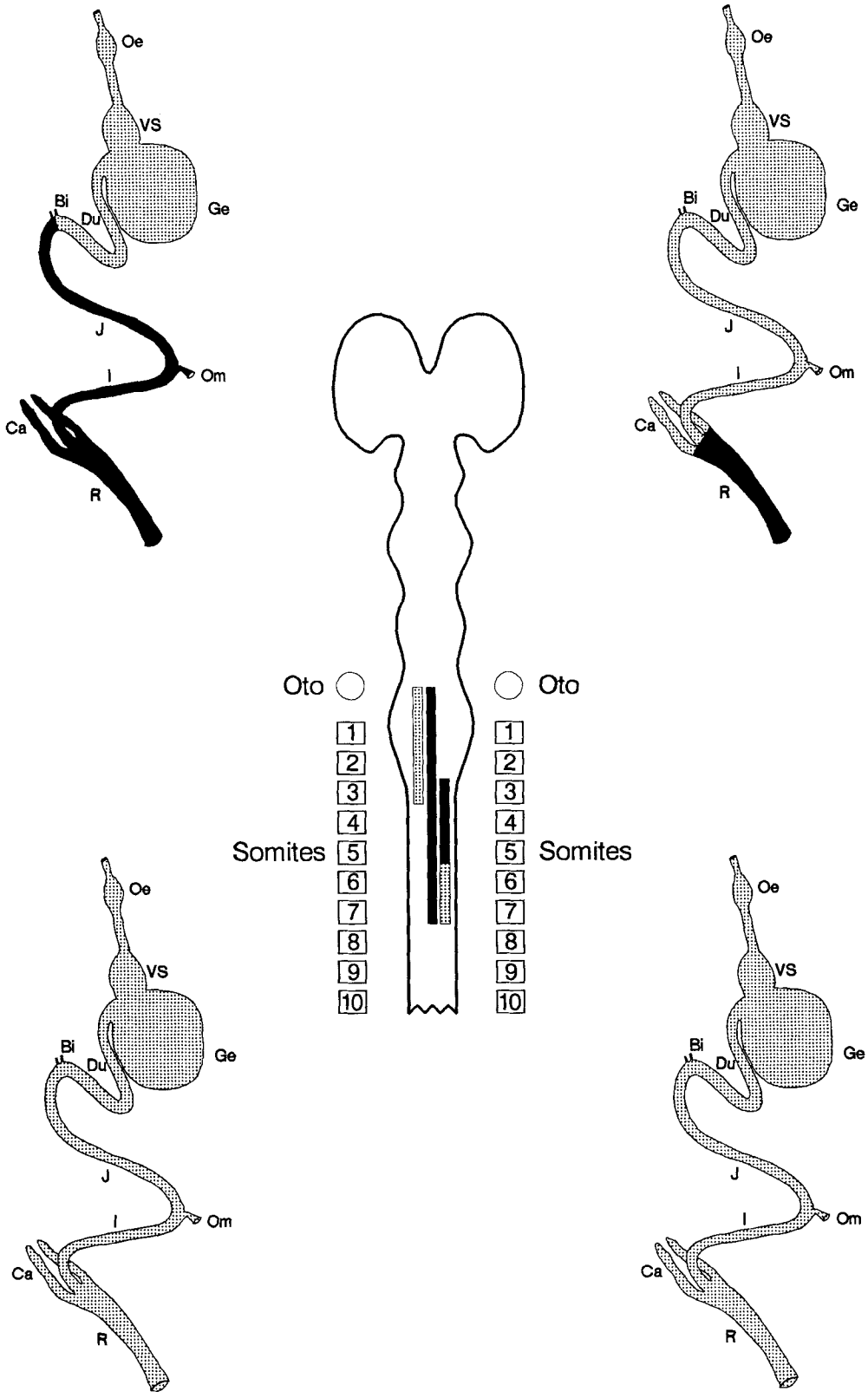

Fig. 4. Schematic drawing summarizing the results of the various neural crest ablations. The bars in the embryo indicate the ablated segment of the neural crest. The parts of the gut coloured black indicate the extent of the aganglionosis, the hatched parts are normally innervated. **Top left:** ablation of MO-S7; **top right:** ablation of S3–5; **bottom left:** ablation MO-S3; **bottom right:** ablation S6–7. Oe: esophagus, Vs: proventriculus, Ge: gizzard, Du: duodenum, Bi: 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

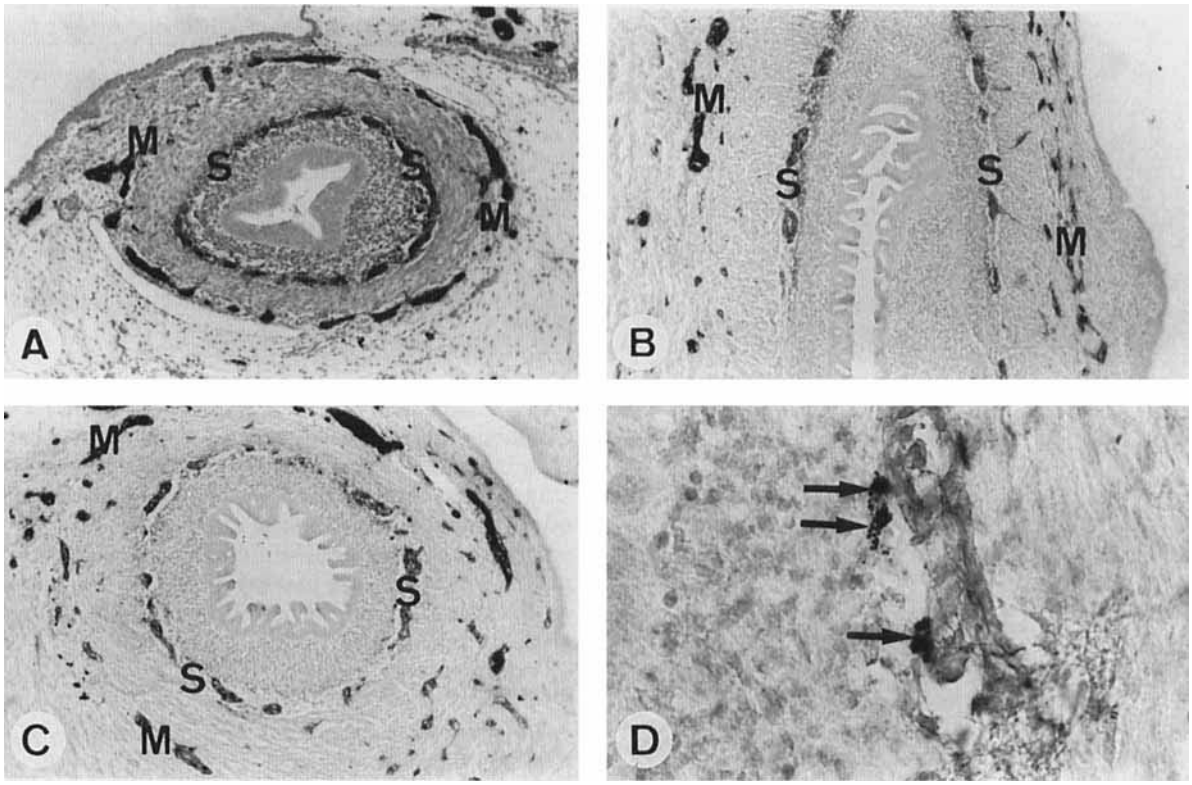

Fig. 5. Paraffin sections of cocultures of parts of quail E2 neural anlage and E4 chicken hindgut stained with the HNK-1 antibody to show the presence of myenteric (M) and submucous (S) ganglia. **A:** Neural anlage from the level of somites 1–3. **B:** Neural anlage from the level of somites 4–7. **C:** 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,

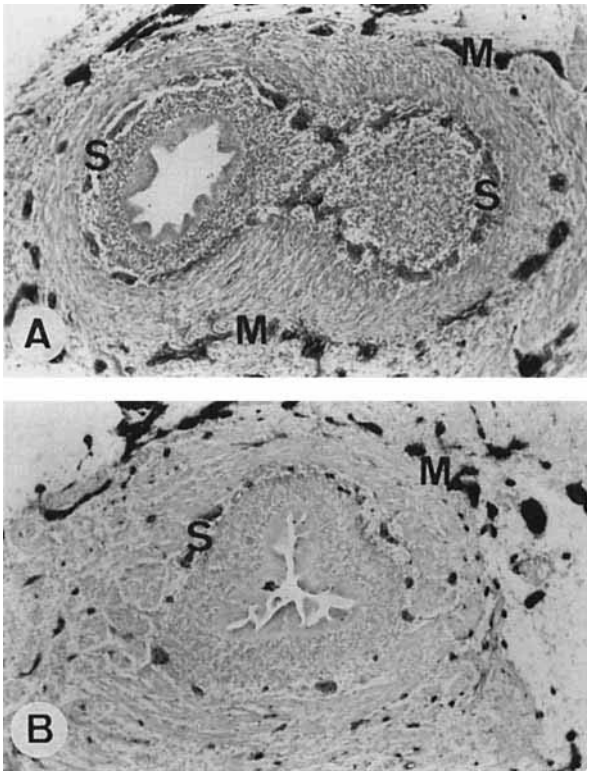

Fig. 6. Paraffin sections of cocultures of quail vagal neural anlage and E4 chicken hindgut. **A:** Vagal neural crest obtained from an embryo with 28 somites: HNK-1 immunoperoxidase staining shows the presence of a normal amount of myenteric (M) and submucous (S) ganglia. ×25. **B:** Vagal neural crest 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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