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THE NEURAL CREST AND NEURAL CREST DEFECTS

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The neural crest is a fascinating embryonic tissue for more than one reason. In the adult organism it gives rise to an array of distinct cell types and tissues. It is responsible for many birth defects, familial diseases and malignancies, and it is amenable to the elucidation of mechanisms that regulate stem cell differentiation. Subsequent to an epithelial-to-mesenchymal transformation, neural crest cells emigrate from the dorsal aspect of the neural tube into the embryo, stop in different places, and eventually give rise to the autonomic and enteric nervous systems, most primary sensory neurons, endocrine cells, and melanocytes of the skin and internal organs. Furthermore, neural crest cells are involved in the septation of the cardiac outflow tract and they form the cranial mesenchyme, which gives rise to bone, cartilage, and connective tissue of the face and ventral neck. Environmental insults can lead to neural crest defects, including cleft lip/cleft palate and fetal alcohol syndrome. Familial diseases that affect neural crest derivatives include Hischsprung's disease and albinism, whereas well-known neural crest-related malignancies include melanoma, neuroblastoma, neurofibromatosis and pheochromocytoma. Migratory neural crest cells form a heterogeneous population of cells that includes stem cells, cells with restricted developmental potentials, and cells that are committed to a particular lineage. Growth factors play important roles in the survival, proliferation and differentiation of neural crest cells. In particular, neurotrophin-3 (NT-3), the ligand of the tyrosine kinase receptor, TrkC, promotes the survival of proliferating neural crest stem cells. TrkC-deficient mice develop cardiac outflow tract defects that resemble human birth defects, including persistent truncus arteriosus and transposition of the great vessels. In these animals, cardiac neural crest stem cells become fate-restricted precociously. Action of stem cell factor (SCF), the ligand of the tyrosine kinase receptor c-kit, affects multiple systems. Heterozygous c-kit *deficient mice, termed 'Dominant spotting' (W), have anemia, are sterile and show changes in coat color (white spotting) due to defects in the hemopoietic system, germ cell line and melanogenesis, respectively. Inactivation of the human* c-kit *gene causes piebaldism, which is characterized by a white forelock, patchy hypopigmentation of the skin and rare sensoryneural deafness. In the quail neural crest, SCF supports the survival of neural crest stem cells, promotes their differentiation into small diameter sensory neurons, and, together with a neurotrophin, supports survival of melanocyte precursors. In* c-kit *deficient newborn mice, up to one third of substance P-immunoreactive nociceptive sensory neurons are missing, thus confirming across species that SCF signaling is essential for the development of small diameter sensory neurons. In addition, the number of calcitonin gene-related-peptide (CGRP)-immunoreactive putative visceral afferent neurons in the dorsal root ganglion is diminished in these mice. The norepinephrine transporter (NET) is expressed in many embryonic tissues, including premigratory and migratory neural crest cells. Norepinephrine (NE) uptake by neural crest cells promotes their differentiation into noradrenergic neuroblasts* in vitro*. In contrast, NE uptake inhibitors, such as tricyclic antidepressants and the drug of abuse, cocaine, inhibit noradrenergic differentiation* in vitro *and* in vivo*, suggesting that these drugs can be teratogenic. Since NET is expressed in many embryonic tissues, NE transport may have functions also in non-neural cells during embryonic development. In summary, growth factors, alone and synergistically, as well as NE play multiple roles in neural crest development.*
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INTRODUCTION

Much like a secondary gastrulation, neural crest cells leave the ectoderm and migrate into the embryo. The mechanisms that regulate this epithelial-to-mesenchymal transformation are currently being elucidated. Neural crest formation is induced by interactions between neural plate and somatic ectoderm. Wnt6 is localized in the ectoderm of the avian embryo, and its inhibition perturbs neural crest formation, suggesting that Wnt6 protein induces neural crest cell formation in the avian embryo (1). mRNA of Noelin-1, a secreted factor, is expressed in a graded pattern in the neural plate with its highest concentration in the neural folds. Its overexpression causes an excess of neural crest emigration and prolongs the time during which neural tube is competent to form neural crest cells. This observation suggests that Noelin-1 is involved in rendering the neural tube competent to respond to inductive signals that generate neural crest (2). Wnt1 and Wnt-3a are involved in dorsal patterning of the neural tube, regulating the expansion of dorsal neural precursors (3,4). In Xenopus, Wnt proteins synergize with Noggin, an inhibitor of bone morphogenetic protein-4 (BMP4), to generate the neural crest as assessed by a dramatic increase in the expression of slug, an early neural crest cell marker (4). In the avian embryo BMP4 mRNA is homogeneously distributed along the longitudinal extent of the dorsal neural tube, whereas its inhibitor, Noggin, is expressed in a rostrocaudally decreasing concentration gradient (5). Experimental increase of local Noggin concentration delays neural crest emigration, suggesting that BMP4 action is involved in neural crest emigration (5). Furthermore, when injected into the lumen of the closed neural tube, Noggin represses neural crest formation, as measured by a decreased expression of the transcription factor, slug (6). In the mouse, Noggin is not essential for neural induction, but is required for the subsequent growth and patterning of the neural tube (7). With the caveat that there might be differences between species, and that some issues remain to be resolved, current understanding is that crosstalk between somatic ectoderm and the neural plate induces the formation of neural crest cells. Early BMP action induces expression of Wnt proteins in the neural tube. Subsequently, Wnt proteins in synergy with BMP antagonists induce neural crest formation, whereas late BMP action may be involved in promoting delamination of neural crest cells from the neural tube.

Neural crest cells migrate *via* different pathways into the embryo and give rise to all neuronal and non-neuronal cells of the autonomic and enteric nervous systems, most primary sensory neurons, to endocrine cells (adrenal medulla, C-cells of the thyroid), and to the pigment cells (melanocytes) of the skin and internal organs. Moreover, neural crest cells contribute to the septation of the cardiac outflow tract and they form the cranial mesenchyme, which gives rise to dermis, bone, and cartilage of the face and ventral neck (described in ref. 8).

The early migratory neural crest forms a heterogeneous population of cells with regard to developmental potentials. At one extreme, there are neural crest stem cells that can give rise to most or all known neural crest derivatives (9-15). At the other end of the spectrum are various types of progenitor cell that are committed to a particular cell lineage (9-15). Furthermore, there are pluripotent neural crest cells that are more restricted in their developmental potentials than stem cells (9-15).

Neural crest stem cells are descendants of neuroepithelial stem cells, which reside in the neural tube. Through instructive action of BMP 2/4, which does not require cell proliferation, neuroepithelial cells become $p75^{NTR}$-immunoreactive and can differentiate into neural crest cells (16). The existence of pluripotent progenitors was shown by *in vitro* clonal analysis (9,11-15) and by labeling individual neural crest cells *in vivo* (10). In both approaches it was found that an individual neural crest cell can give rise to an array of differentiated progeny, including sympathetic neurons, sensory neurons, nerve supporting cells, pigment cells, smooth muscle cells, chondrocytes, fibroblasts and possibly other cell types (14). Part of the definition of a stem cell is not only that it can generate one or more types of progeny but also that it is capable of generating new stem cells (self-renewal). The potential for self-renewal of rat neural crest stem cells was shown first by serial cloning *in vitro* (17).

During advanced migration, the developmental potentials of neural crest cells become increasingly more restricted. For instance, cultured cardiac neural crest stem cells that have been obtained from the neural tube can generate at least six phenotypes (14). In contrast, once they have migrated through the posterior branchial arches and have arrived in the cardiac outflow tract, they have lost the capacity to give rise to sensory neurons and to melanocytes (15).

Neural crest stem cells persist in target locations. By *in vitro* clonal analysis we have shown that pluripotent progenitors are present in the somatic ectoderm (18), dorsal root ganglia (19), sympathetic ganglia (19) and cardiac outflow tract (15). Recently stem cells have also been identified in the embryonic sciatic nerve and gut (20,21)

Neural crest progenitor cells change their properties in different target locations. Both, multipotent cells isolated from dorsal root ganglia and multipotent cells from sympathetic ganglia, can give rise to pigment cells, sensory neurons, sympathetic neurons and possibly other cell types *in vitro*. However, under the same culture conditions, progenitors from dorsal root ganglia generate 10 times more sensory neurons than sympathetic neurons. Conversely, progenitors from sympathetic ganglia of the same embryo give rise to 10 times more sympathetic neuroblasts than sensory neurons when cultured under the same conditions (22). This observation indicates that the embryonic microenvironment modulates the probability of stem cells to generate a particular progeny cell

type by creating a bias for the emergence of site-appropriate cells.

DELETION OF THE TrkC GENE CAUSES A CARDIAC NEURAL CREST STEM CELL DEFECT

Cardiac neural crest cells originate from the neural tube segment that extends from the midotic placode to somite 3 axial levels. They migrate through the posterior branchial arches to the cardiac outflow tract and the proximal great vessels (23,24). Ablation of the cardiac neural crest in chick embryos (25,26) results in cardiac outflow tract defects that are similar to those seen in neurotrophin-3 (NT-3) null and TrkC null mutants (27,28), including persistent truncus arteriosus and transposition of the great vessels.

Bound NT-3 activates TrkC kinase, leading to the recruitment and phosphorylation of signaling proteins, which regulate neuronal proliferation, differentiation and survival (29). TrkC null mice have a phenotype that is similar to that of NT-3-deficient mice, confirming that NT-3 acts predominantly through TrkC (30). Alternative splicing of the TrkC gene can generate several types of truncated TrkC isoforms, which lack the kinase domain (31-33). These noncatalytic receptors, which are well-conserved among species, are thought to negatively modulate signaling by the catalytic receptor when both the catalytic and truncated isoforms are co-expressed (34,35). Both, catalytic and truncated TrkC receptors were absent in the TrkC null mouse line that was used in our study (27). In contrast to other Trk receptors, TrkC is widely expressed in many different tissues of the mouse embryo, including muscle, lung, kidney, heart, and vascular smooth muscle cells (28,36). The wide expression pattern suggests that NT-3 has pleiotropic functions during embryonic development (36), including mitogenic action in cardiac myocytes during cardiac looping and establishment of ventricular trabeculation (37).

To elucidate the mechanisms that lead to neural crest-related cardiac outflow tract defects in TrkC null mice, we have performed *in vitro* clonal analysis of cardiac neural crest (CNC) cell development. We have identified three types of progenitor cells, stem cells (CNC-SC), fate-restricted cells (CNC-RC), and cells that are committed to the smooth muscle lineage (CNC-smC)(38). Stem cells are capable of self-renewal and can give rise to at least 5 differentiated phenotypes, including smooth muscle cells, neurons, Schwann cells, chondrocytes and pigment cells. In contrast, fate-restricted cells generate no neurons and no pigment cells; chondrocytes and Schwann cells are observed rarely only. CNC-smC cells give rise to smooth muscle cells only. The expansion potential, as measured by *in vitro* colony assay, differs between the different progenitor cells at a ratio of approximately CNC-SC : CNC-RC : CNC-smC = 35 : 10 : 1. Thus, stem cells proliferate in culture up to 35 times faster than progenitors that are committed to the smooth muscle cell lineage. Approximately 77% of early migratory colony-forming cardiac neural crest cells are of the CNC-smC type, that is they are committed to the smooth muscle cell lineage. CNC-RC form 37% of the population, whereas stem cells comprise approximately 15%. In TrkC null mice, the portion of stem cells is decreased approximately by half to 8%, whereas the portion of fate-restricted cells is increased by approximately 50%. The portion of fate-restricted cells is unchanged. The loss of half of all stem cells and the equivalent increase of fate-restricted cells suggests that stem cells become restricted in their developmental potentials prematurely. Since we were analyzing early migratory neural crest cells, our data provide a stationary picture only, whereas in reality it is a dynamic process that may continue during *in vivo* migration. Thus the stem cell loss is likely to be greater in the population of neural crest-derived cells when they arrive in the outflow tract. The precocious differentiation of stem cells into fate-restricted cells is likely to lead to at least two changes in outflow tract development. First, since the expansion rate of fate-restricted cells is 3.5-fold lower than that of stem cells, the final number of neural crest-derived cells present in the TrkC null outflow tract may be reduced compared to the wild type. Second, there is an inverse relationship between migratory capacity and state of differentiation, which may lead to premature cessation of migration. In fact, our observations *in vivo* support this notion. In sections through the TrkC null outflow tract area, a large number of neural crest cells is present, as in the wild type, and no apoptotic cells are detected. However, in double stains with the neural crest marker, 4E9R (39), which is down-regulated during differentiation, and antibodies against smooth muscle actin, it becomes apparent that in TrkC null outflow tract cells are predominantly smooth muscle actin-immunoreactive and 4E9R negative. Moreover, they tend to remain within the wall of the outflow tract, rather than undergo radial migration that leads to the septation of the outflow tract into the aorta and pulmonary artery (38).

INACTIVATION OF THE C-KIT GENE CAUSES A LOSS OF SMALL AND MEDIUM DIAMETER SENSORY NEURONS IN THE DORSAL ROOT GANGLION

c-kit is a tyrosine kinase receptor (40), which binds stem cell factor (SCF). Mice with a deleted *c-kit* gene ('Dominant spotting' or W mouse) or deleted *SCF* gene (Steel mouse), have three major defects. They include change in coat color, anemia and sterility. W/+ mice survive and appear normal except for white spotting of the coat (Fig. 1**a**, **b**). W/W mice are significantly smaller than normal litter mates, they are pale and die perinatally (Fig. 1**c**). In W/W mice the c-kit receptor is truncated due to a single base mutation, which causes the deletion of 78 amino acids that include the transmembrane domain and N-terminal amino acids of the kinase domain (41). The resulting 125 kD intracellular protein is therefore not expressed at the cell surface and has no c-kit

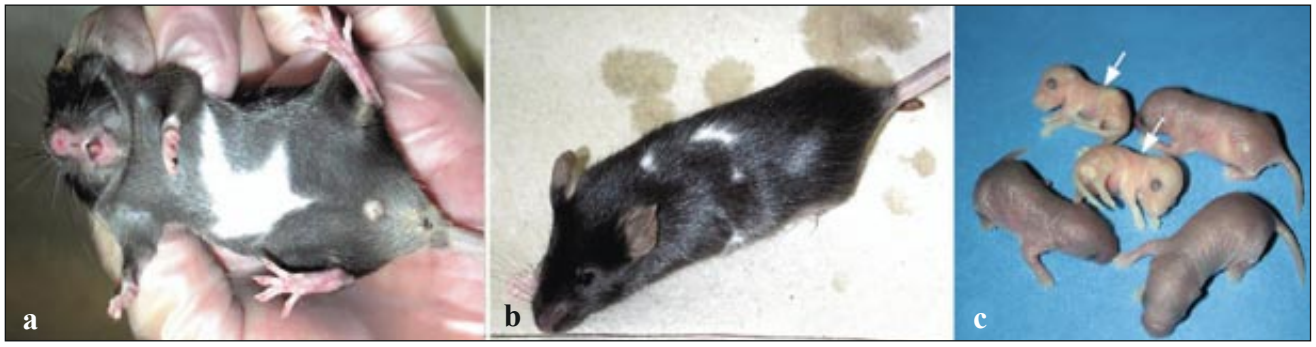

Figure 1. *Phenotype of W/+ and W/W mice. W/+ have a characteristic abdominal white spot* ***(a)*** *and variable white spots on the back* ***(b)****. W/W newborns (****c****, arrows) appear pale and are significantly smaller than wild type (wt) litter mates (from reference 56).*

kinase associated activity (42). When c-kit is expressed in COS cells, c-kit shows constitutive tyrosine phosphorylation and low association with phosphatidylinositol (PI)-3'-kinase and phospholipase-Cγ1. Upon binding of soluble SCF, the receptor dimerizes and both autophosphorylation and binding of secondary messenger proteins increases (43). The human *c-kit* gene consists of 21 exons that are distributed over more than 70 kilobases at chromosome segment 4q12, adjacent to the closely related platelet-derived growth factor receptor (44-46). Mutations in the human *c-kit* gene, termed piebaldism, are characterized by congenital patches of white skin (leukoderma) and white hair (poliosis) located primarily on the scalp, forehead, ventral chest and abdomen (44), and rare sensoryneural deafness (47).

SCF has a three-fold impact on quail neural crest cell development. One, SCF is a survival factor for the neural crest stem cell. Two, SCF promotes differentiation of neural crest stem cells into sensory neuroblasts. Three, SCF together with a neurotrophin of the nerve growth factor (NGF) family, supports the survival of cells that are committed to the melanocyte lineage (48). All three neurotrophins, NGF, brain-derived neurotrophic factor (BDNF) and NT-3 are equally effective, suggesting that they act through the non-selective neurotrophin receptor, $p75^{NTR}$ (48).

SCF and c-kit are expressed in small and medium diameter neurons of the murine dorsal root ganglion (49-51). Approximately 44% of c-kit-positive sensory neurons contain substance P (SP)(52). SP-immunoreactivity is correlated with nociception (53). Calcitonin gene-related-peptide (CGRP)-immunoreactivity in lower lumbar regions is associated with visceral afferents, vasculature and motor endplates of striated muscle, bone, joint capsules, cutaneous neurons with free nerve endings, and fibers that innervate dermal blood vessels and sweat glands (54,55).

Due to the multiple effects of SCF in quail neural crest cell development and the known expression of c-kit and SCF in mouse dorsal root ganglia, we sought to analyze the effect of nonfunctional c-kit in the mouse dorsal root ganglion. To this end we have developed a PCR-based genotyping protocol for the W mutation (56). W/W live newborns were sacrificed, genotyped, dissected and frozen sections prepared from the spinal cord with adjacent L6 dorsal root ganglia. The tissue was processed for indirect immunocytochemistry with antibodies against beta-III tubulin (neuronal marker), SP (marker for small diameter sensory neurons), and DAPI nuclear stain for total cell counts (Fig. 2). Alternatively, antibodies against CGRP were used to detect small and medium diameter sensory neurons. The total number of cells (DAPI stain), neurons (beta-III tubulin stain) and SP-immunoreactive, or CGRP-immunoreactive, neurons *per* area were scored. The area was measured with Metamorph software (Universal Imaging) and the data expressed as number of cells *per* area. A proportion of 24% of DRG neurons were SP-immunoreactive, while 35% of neurons were CGRP-immunoreactive. The total number of neurons was not affected detectably. In contrast, the number of SP positive cells was decreased significantly by approximately 33%. There was a smaller, less pronounced decrease in the number of CGRP-immunoreactive neurons (56). Our data suggest that SCF signaling through c-kit is essential for the development or survival of some nociceptive and visceral sensory neurons. It is conceivable that the neuronal loss is aggravated in postnatal life. This cannot be tested, however, as the animals die shortly after birth.

NOREPINEPHRINE TRANSPORT PROMOTES NORADRENERGIC DIFFERENTIATION

The norepinephrine transporter (NET) is a channel-like transmembrane protein that is characteristically localized in adult noradrenergic neurons of the sympathetic nervous system and the locus ceruleus (57,58). In the mature nervous system, its function is to remove released norepinephrine from the sy-

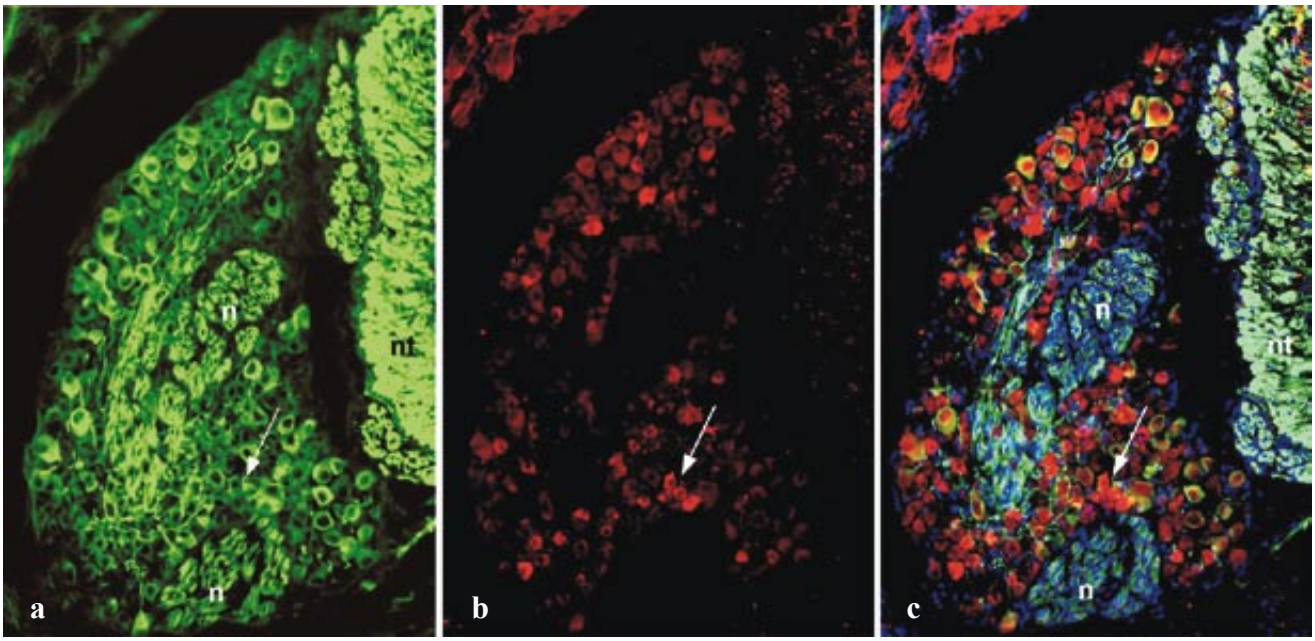

Figure 2. *Wild type L6 dorsal root ganglion. Triple stain.* ***(a)*** *β-III tubulin immunoreactivity visualizes all neurons, nerve fibers (n). The adjacent neural tube (nt) is also strongly immunoreactive.* ***(b)*** *substance P-immunoreactivity (red). A group of 3 cells is prominently substance P-positive (arrow).* ***(c)*** *Merged images of β-III tubulin (green), substance P (red) and DAPI nuclear (blue) stains. Areas that are predominantly blue are bundles of neurites (n) in cross section. nt, neural tube (from reference 56).*

naptic cleft (59-61). The same *NET* gene product is expressed in the central and peripheral nervous systems in the *locus ceruleus*, sympathetic neurons and the adrenal gland (57).

NET has additional roles during embryonic development. It is expressed very early in both avian embryos and mammalian embryos (62,63). NET mRNA levels are increased synergistically by autocrine NT-3, transforming growth factor-β1 and fibroblast growth factor-2 action (62). NE transport promotes differentiation of neural crest cells (64) and *locus ceruleus* cells (65) into noradrenergic neurons. Conversely, drugs that block NE uptake, such as tricyclic antidepressants or cocaine, block noradrenergic differentiation *in vitro* and *in vivo* (64-68). Quail and mouse neural crest cells express NET protein even before onset of their emigration from the neural tube (63). While at that stage the transporter may not yet be functional, it is known that neural crest cells are capable of NE transport during advanced migration, when they arrive in the vicinity of the notochord (69). Interestingly, the avian notochord has been shown to be able to synthesize NE and other neurotransmitters before the emergence of any differentiated neurons in the central or peripheral nervous system (70). In the mammalian embryo, neurotransmitters are thought to originate in the maternal blood stream and to enter the fetal bloodstream through the placenta (71).

Inhibition of NET function has adverse effects on noradrenergic differentiation. If neural crest cells are treated with desipramine or cocaine *in vitro* (66-68) or *in vivo* (65), there is a significant decrease in the number of cells that express dopamine-β-hydroxylase in the sympathetic ganglion and in the locus ceruleus. This observation and studies with adrenergic receptor agonists and antagonists (68) confirm that NE transport, rather than activation of adrenergic receptors, is responsible for promoting noradrenergic differentiation. It is known that infants who have been exposed to antidepressants or cocaine prenatally have a higher incidence of sudden infant death syndrome and autonomic disturbances, which appear to be due to a delayed maturation of the noradrenergic system in the *locus ceruleus* and the autonomic nervous system (72,73).

NET and NE transport, as well as other transporters, such as the serotonin transporter (SERT), may have an even wider impact on morphogenesis during embryonic development. NET is not only expressed in neurons and their progenitor cells, but in many parts of the avian and mammalian embryos. Neuronal and non-neuronal tissue derived from all three germ layers express NET during quail and mouse embryonic development (63). Potentially important sites include the brain, spinal cord (floor plate, ventral horn), the cardiovascular system (heart,

walls of arteries and veins, endothelial cells), the kidneys, lungs and intestinal tract. One common denominator of NET expressing cells is that they either form an epithelium or are derived from an epithelium (63). Lauder and collaborators have shown that serotonin promotes neural crest cell migration during craniofacial development, and that inhibition of serotonin transport causes craniofacial malformations (71,74). Since many epithelial cells express NET or SERT, and many underlying mesenchymal cells express adrenergic or serotonergic receptors, we have proposed that NET and SERT may function as a targeted delivery system of the neurotransmitters (63). Both neurotransmitters are present in high concentrations in fetal serum (67). Epithelial cells exposed to blood or other fluids may take up the neurotransmitter and accumulate it. If the concentration gradient exceeds a certain threshold, that transporter may reverse and release the neurotransmitter into the subjacent mesenchymal space. Neurotransmitter receptors, which are expressed by many mesenchymal cells (71,75) may in turn be activated (summarized in Fig. 3). Activation of norepinephrine or serotonin receptors expressed by embryonic cells can affect many growth processes, including cell division (76,77), apoptosis (78) and cell differentiation (79).

Taken together, neurotransmitter transporters are widely expressed during embryogenesis. Neurotransmitters and their corresponding transporters and receptors appear to have multiple functions during morphogenesis. This implies that inhibition of neurotransmitter transport perturbs morphogenesis. In two cases evidence has been provided. They include craniofacial defects due to SERT blockage (71) and delayed differentiation of noradrenergic systems by blockage of NET (65).

CONCLUSION

Neural crest progeny are critical elements in the formation of the peripheral nervous system, the cardiac outflow tract and craniofacial features. The wide distribution of neural crest cells in the organism and their high rate of proliferation make neural crest progeny vulnerable to environmental insults and genetic defects. Growth factors play important roles in the migration, survival, proliferation and differentiation of neural crest cells. Mutations that affect pertinent growth factors or their receptors lead to neural crest-related birth defects or familial diseases. Two of them have been discussed here. TrkC null mice develop cardiac outflow track defects that resemble human birth defects. They include persistent *truncus arteriosus* and transposition of the great vessels. Lack of NT-3 signaling through TrkC causes a cardiac neural crest stem cell defect in mice that leads to a precocious fate restriction of the stem cell. As a consequence, the total number of neural crest cells in the TrkC null outflow tract and cell mobility during conotruncal septation may be decreased. Stem cell factor signaling through c-kit has multiple effects in several neural crest lineages. It supports survival of neural crest

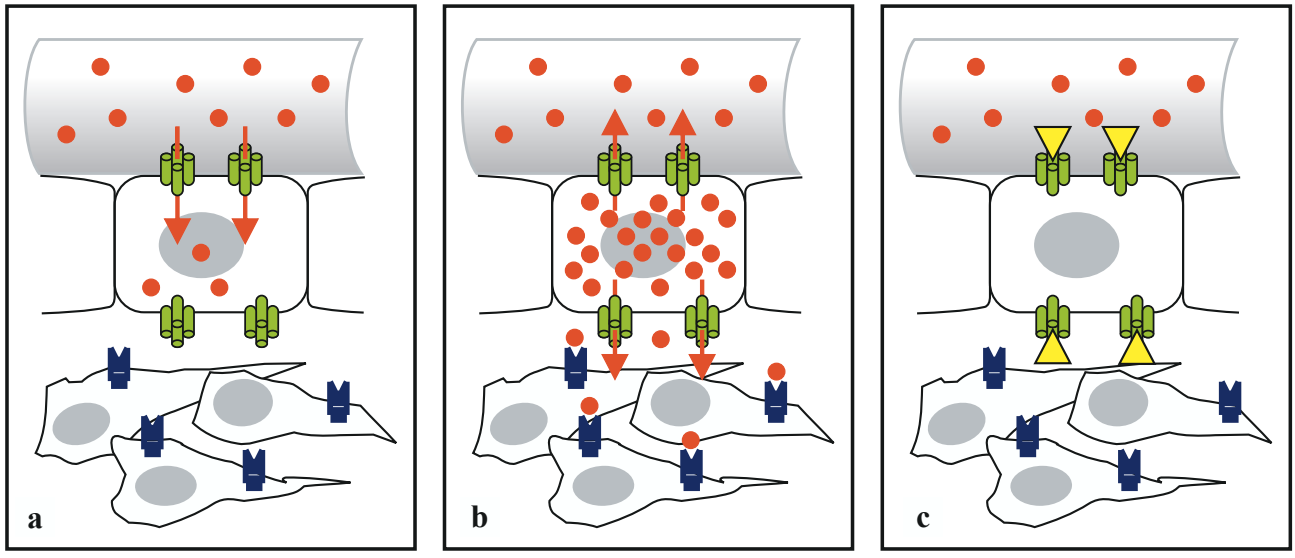

Figure 3. *Model for the role of NET in embryonic morphogenesis.*
(a) *Norepinephrine (●) in blood, or other fluid, is transported by epithelial cells that express NET ( ). **(b)** When the amine gradient reaches a critical threshold, NET reverses and releases NE to the subjacent mesenchymal cells. Mesenchymal cells express adrenergic receptors ( ), which are activated by NE. **(c)** NET inhibitors (△), such as tricyclic antidepressants or cocaine, block both transport and reverse transport. No NE is delivered to the mesenchymal cells that underlie the epithelium (from reference 63).*

stem cells, promotes their differentiation into small diameter sensory neurons, and - synergistically with a neurotrophin (NGF, BDNF or NT-3) - promotes the survival of cells that are committed to the melanogenic lineage. The W mouse, in which c-kit is rendered inactive by a point mutation, is a good model system for human piebaldism. In order to make W/W embryos accessible to experimentation, we have developed a PCR-based genotypic protocol. Furthermore, we have found that in W/W mice a significant portion of small diameter and medium size sensory neurons in the L6 dorsal root ganglion are missing, suggesting that nociceptive and visceral afferent systems are dependent on SCF/c-kit signaling. Environmental insults that affect neural crest cell migration or survival can also lead to perturbations in neural crest-derived tissues. We have shown that norepinephrine transport promotes *in vitro* differentiation of noradrenergic cells both in the neural crest and *locus ceruleus*. Conversely, substances such as tricyclic antidepressants and cocaine, that block norepinephrine transport, inhibit noradrenergic differentiation *in vitro* and *in vivo*. We thus conclude that substances that block norepinephrine transport could be teratogenic. This view is supported by studies, which show that prenatal exposure to these drugs can cause autonomic disturbances and higher incidences of sudden infant death syndrome in newborn infants. Since the norepinephrine transporter is expressed ubiquitously in neuronal and non-neuronal embryonic tissues, norepinephrine transport may play a role in the development of other tissues as well.

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