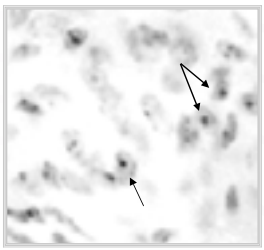
Development of the Enteric Nervous System

Mike Gershon

The ENS is a unique part of the nervous system

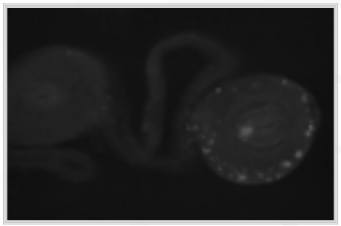
- Mediates behavior of gut in absence of input from CNS.
  - Most neurons not connected to CNS
- Lacks internal collagen
- Support from enteric glia
- Many neurons and many types of neuron
  - Every class of neurotransmitter found in CNS is also in ENS
  - More neurons than spinal cord
  - More neurons than remainder of PNS
  - Greatest phenotypic diversity in PNS

Quail-chick interspecies chimeras reveal the migration pathways of crest-derived cells



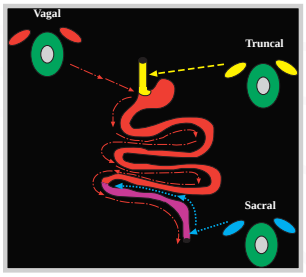
- Chick crest is removed before migration begins.
- Replaced with a graft of quail crest.
  - Quail crest cells migrate in host.
- Quail crest cells are stably marked by their distinctive nucleolus-associated heterochromatin.
- Location of quail cells reveals destinations reached by migrating crest-derived cells.

DiI-labeled sacral crest cells colonize the post-umbilical bowel



- DiI was injected into neural crest of a chick embryo caudal to somite 28.

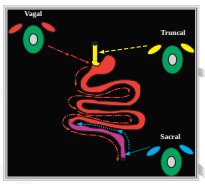
The gut is colonized by precursors that migrate from the neural crest.



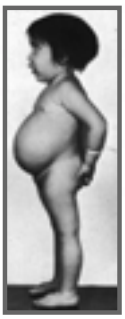
- **Vagal level: whole gut. Anterior → posterior**
- **Truncal level: rostral foregut (Esophagus).**
- **Sacral level: postumbilical gut. Posterior → anterior**

Microenvironmental signals determine the fates of crest-derived cells

- Signals from the environment received by crest cells regulate their:
  - migratory paths
  - proliferation
  - restriction of developmental potential
  - survival
  - formation of terminally differentiated derivatives.
- As crest-derived cells migrate they change:
  - cell surface receptors
  - intracellular transduction mechanisms.
- Postmigratory cells in the gut are thus different from their premigratory precursors in the neural crest.



Congenital aganglionosis causes pseudoobstruction



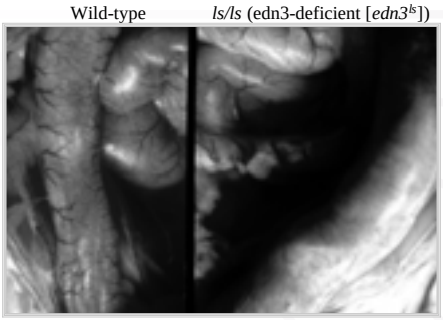
- Hirschspung's disease results from aganglionosis of the terminal colon.
- Associated with the development of megacolon.
- Relatively common disease
  - 1/5000 births in general population
  - 1/500 births in Mennonites (due to inbreeding
- Most commonly due to defect in RET > EDNRB.

Crest-derived cells require Edn3 (ET-3) and Ednrb (ET_B) to complete their colonization of the gut

- The endothelins are vasoactive peptides
  - edn1 (ET-1), edn2 (ET-2), edn3 (ET-3)
- Big endothelins are secreted and converted in tissues to active peptides by endothelin converting enzymes (1 and 2).
- There are 2 endothelin receptors.
- Ednra (ET_A) and Ednrb (ET_B).
  - edn1 and edn2 stimulate both
  - edn3 only activates Ednrb.
    - ENS development requires edn3 and ednrb.

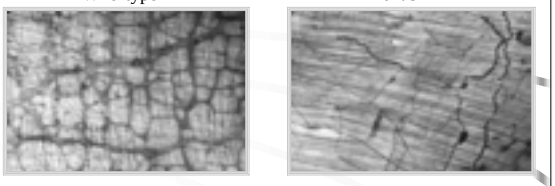
Megacolon occurs in mice that lack edn3 (ET-3)

Wild-type *ls/ls* (edn3-deficient [*edn3^{ls}*])



The terminal colon of ET-3-deficient mice is aganglionic

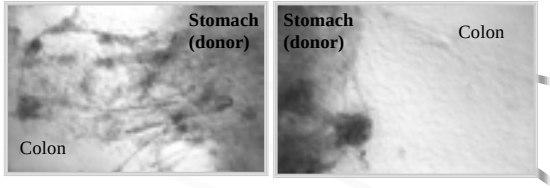
Wild-type *end3^{ls}*



- The aganglionic bowel is not denervated.
  - It contains large nerve trunks containing extrinsic axons and projections from the proximal hypoganglionic bowel.

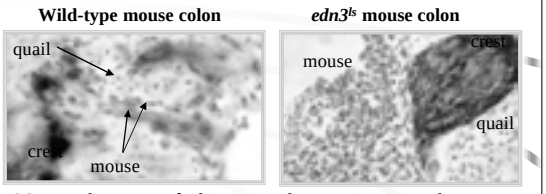
Co-cultured sources of crest fail to colonize presumptive *end3^{ls}* gut

Wild-type mouse colon **_edn3^{ls}_ mouse colon**



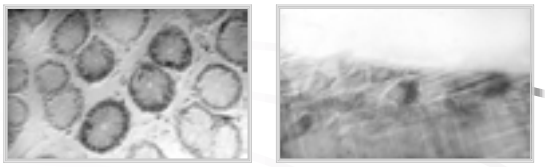
- Donor neurons marked by AChE activity.
- Donor neurons enter wild-type mouse colon but not *end3^{ls}* colon.

Presumptive aganglionic gut from *edn3^{ls}* mice cannot be entered by quail crest cells



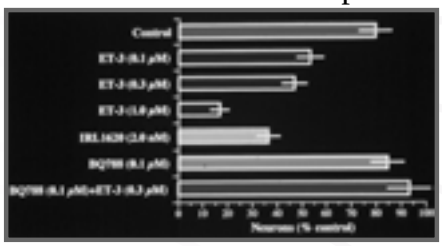
- Mouse colon was grafted into a quail crest migration pathway.
- Crest is immunostained blue (HNK1).
- Mouse nuclei are different from those of quail, enabling a graft of mouse gut to be recognized in a quail host.

The terminal colon is normally colonized in *end3^{ls}* <> WT chimeric mice



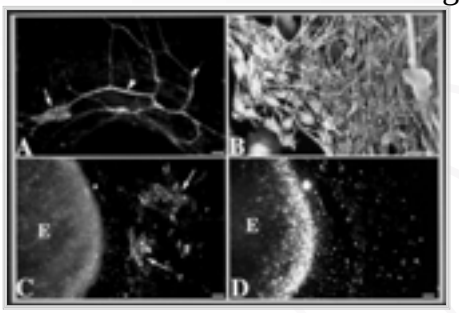
- Cell of WT mice have low and *end3^{ls}* mice have high levels of β-glucuronidase
- Crypts are clonal in origin.
- Neurons and connective tissue cells are either WT or *edn3^{ls}*.
- *Edn3^{ls}* neurons are found in the terminal colon.

Edn3 inhibits the development of neurons from crest-derived precursors

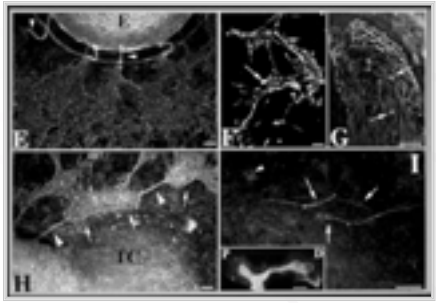


- Edn3 effects are mimicked by the ETB agonist, IRL1620 and blocked by the antagonist BQ788, but neurons develop in the presence of BQ788. Edn3 is not required for neural development.

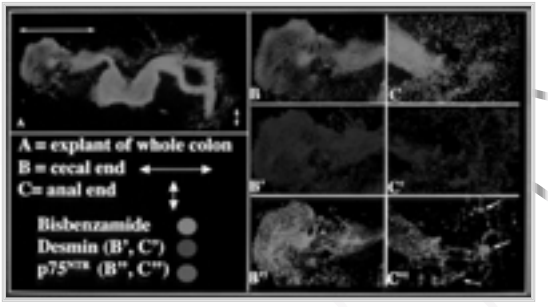
Crest-derived cells are present in the proximal bowel of edn3-deficient mice but do not enter the terminal gut



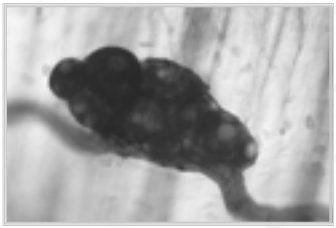
Exogenous Edn3 enables crest-derived cells to enter the terminal colon of Edn3-deficient mice



Exogenous ET-3 allows crest-derived cells to colonize the entire colon *in vitro*

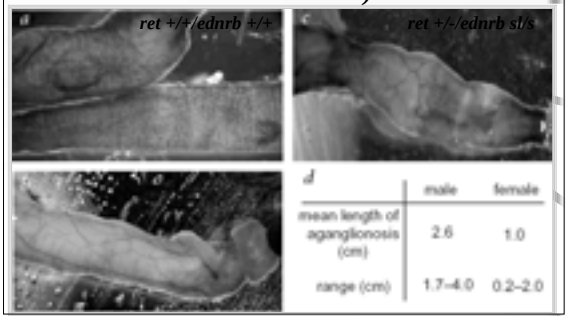


Ectopic ganglia develop in the pelvis of *end^{ls}* mice



- Structure is that of peripheral nerve, not ENS.
- Thought to be derived from sacral crest cells that have stopped migrating before reaching the gut.

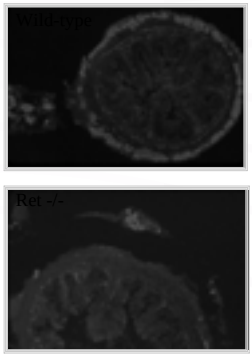
Ret and Ednrb interact in humans and in mice (mice tested to verify human data)



d

	male	female
mean length of aganglionosis (cm)	2.6	1.0
range (cm)	1.7–4.0	0.2–2.0

Enteric neurons are Ret-dependent

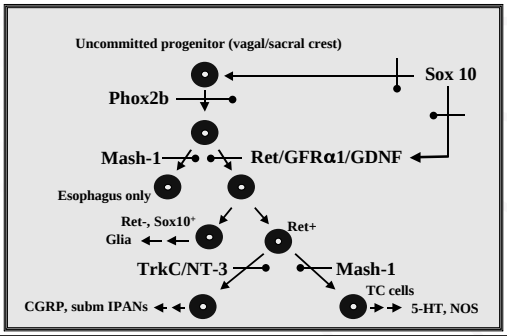


- GDNF binds to GRFα1 and stimulates Ret.
- Mice that lack Ret (or GDNF or GFRα1) lack enteric neurons below the level of the esophagus.
- Loss of function mutations in *RET, GDNF, or GFRα1* are associated with Hirschsprung's disease

The GDNF family of growth factors activate Ret

- Ret is a receptor tyrosine kinase that is expressed in the gut only by crest-derived cells.
- Activated by ligands that bind to co-receptors
- Ret stimulates proliferation early in development, is a chemoattractant for migrating crst-derived cells, and supports survival.

Specific transcription and growth factors define stages in ENS development



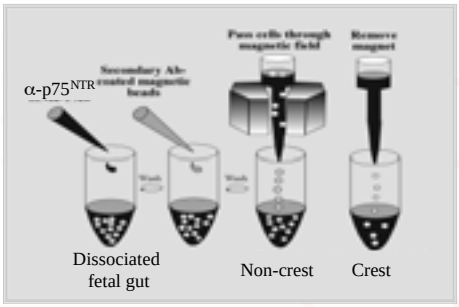
The earlier a gene acts in development, the more massive the defect that follows its deletion

- Genes that lead to complete aganglionosis when knocked out
  - Phox2b
  - Sox10
  - Ret/GDNF;GFRa1 (below esophagus)
- Genes that lead to limited lesions when knocked out
  - Mash-1
  - Edn3/Ednrb
  - NTN/GFRα2
  - NT-3/TrkC

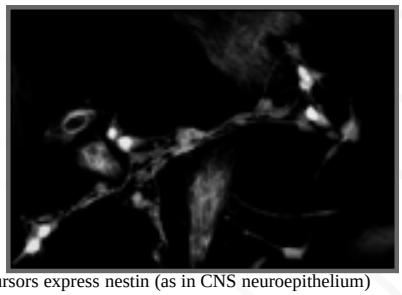
Genes associated with Hirschsprung's disease

- Phox2b: Transcription factor expressed by the most primitive of the crest-derived cells that colonize the gut.
- Sox10: Transcription factor: required early in development.
- Ret, its co-receptors, and ligands: Receptor tyrosine kinase activated first by GDNF, and then NTN.
- EDN3 and EDNRB: collaborates with Ret and needed by non-crest-derived cells of colon
- SIF1: Encodes Smad protein, involved in BMP signaling

Crest-derived cells are isolated by immunoselection.



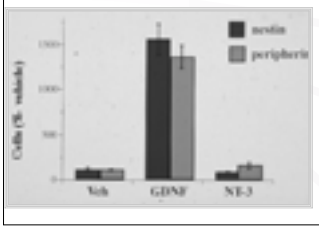
Neurons develop in cultures of isolated crest-derived cells.

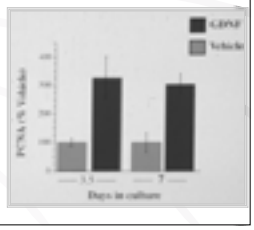


- Precursors express nestin (as in CNS neuroepithelium)
- Neurons express PGP9.5 (a neuronal form of ubiquitin hydrolase).

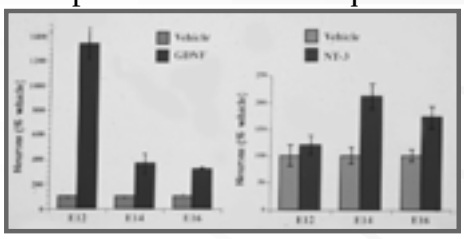
GDNF is mitogenic and promotes neurogenesis at E12

- GDNF increases precursors (**nestin**) and neurons (**peripherin**)
- NT-3 affects neither.
- **GDNF** increases proliferating (PCNA-expressing) cells .



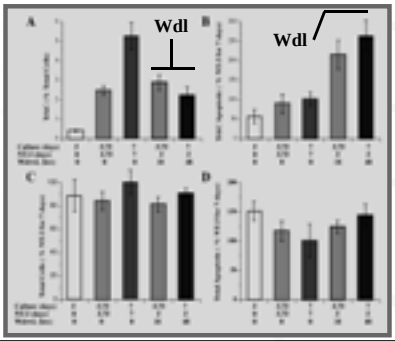


Promotion of neurogenesis by GDNF decreases at E14; neurogenic response to NT-3 is acquired



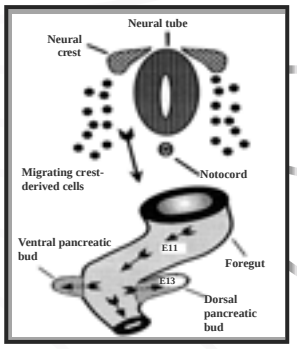
- Even at later ages, the neurogenic response to GDNF is greater than that to NT-3.

Withdrawal of NT-3 leads to apoptosis in TrkC-expressing neurons



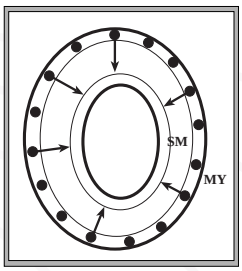
Crest-derived cells colonize the bowel and then migrate from the gut to the pancreas.

- **Vagal crest-derived cells remain in the foregut while pancreatic buds form.**
- **At E13 they enter the pancreas.**

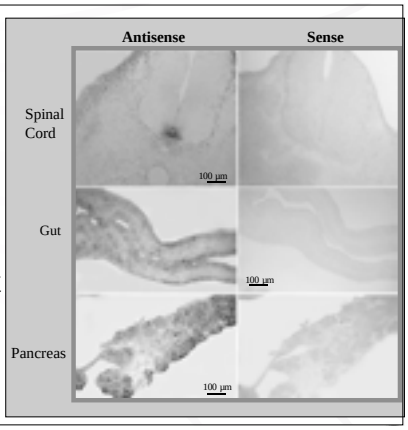


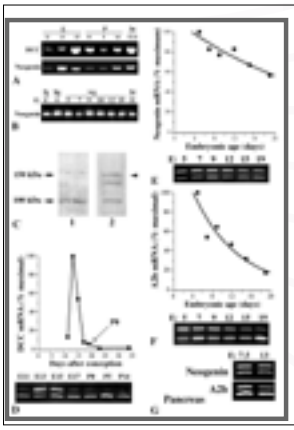
Crest-derived cells migrate in the outer gut mesenchyme; the submucosal plexus forms secondarily.

- **Vagal crest-derived cells migrate proximo-distally down the bowel in its outer mesenchyme.**
- **At E13 subsets of these cells migrate into the submucosa.**
  - **This pattern is true for the entire bowel in the mouse and for the small intestine in the chick.**



mRNA encoding netrin-1 is found in E13 mouse gut and pancreas.

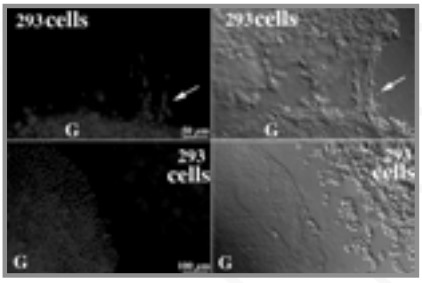




Transcripts encoding netrin receptors are expressed in the developing gut and pancreas

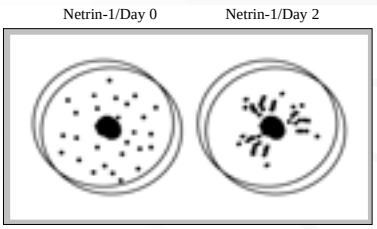
- **DCC, neogenin, and A2b adenosine.**
  - **DCC immunoreactivity found by Western analyses**
  - **Protected by caspase and metalloprotease inhibitors**
- DCC expression is developmentally regulated.
- Found in mouse and chick.

Crest-derived cells migrate from explants of bowel toward transfected cells expressing netrin-1.



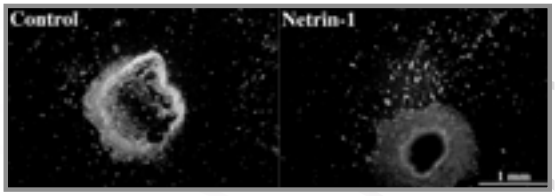
- Enteric cells do not migrate toward control, non-transfected cells.
- Netrin-1 has a chemoattractive effect on crest-derived cells in the gut explant.

Crest-derived cells migrate toward cells that secrete netrin-1.



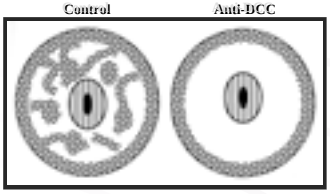
- Immunoselected E6 chick crest-derived cells were plated over a clump of HEK 293 Netrin-1 secreting cells and grow for 2 days

Crest-derived cells isolated from chick gut migrate toward co-cultured netrin-1-expressing cells.



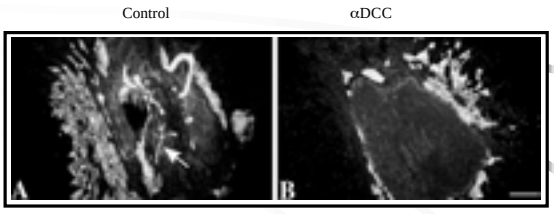
- Stably transfected cells expressing netrin-1 were embedded in 3-D collagen gels. Immunoselected crest-derived cells were plated over the gels.
- Netrin-1 has a chemoattractive effect on immunoselected enteric crest-derived cells in vitro.

Crest-derived cells move internally from the outer gut mesenchyme in explanted rings; this translocation is blocked by anti-DCC.



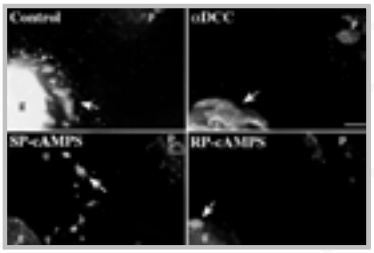
- *Crest-derived cells migrate in the outer gut mesenchyme (ringing the bowel); after the formation of the myenteric plexus an inward migration occurs to form the submucosal plexus.*

Antibodies to DCC block the inward migration of crest-derived cells in chick gut explants.



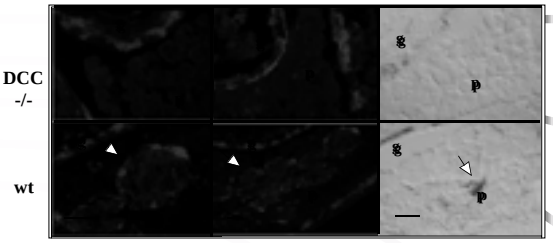
- Crest-derived cells were identified with anti-HNK-1.
  - Bar = 100 µm.

Antibodies to DCC inhibit the migration of crest-derived cells from gut toward pancreas.



- **E5 chick gut and pancreas were co-cultured.**
- **Crest-derived cells migrate out of gut toward pancreas.**
  - **This migration is inhibited by α-DCC and RP-cAMPS, but not by SP-cAMPS.**
- **These observations suggest that pancreatic netrin attracts enteric crest-derived cells through DCC *in vitro*.**

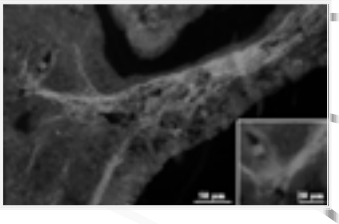
The pancreas of DCC -/- mice is aganglionic

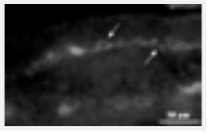


- Neurons (PGP9.5- or acetylcholinesterase-labeled) were found in wt but not DCC-/- mice at P0 (g = gut; p = pancreas; bar = 100 µm).
- Netrin/DCC play an important role in directing the migration of crest-derived cells into submucosa and pancreatic bud in vivo.

Vagal axons entering the stomach at E13 are DCC-immunoreactive

- At E13, vagal fibers reach the esophago-gastric junction and extend growth cones into the wall of the stomach (right).





- At E13, crest-derived cells migrate out of the stomach into the duodenum (left).
  - See Poster by Ratcliffe et. al. - this meeting.

Summary & Conclusions

- The ENS is derived from a multipotent set of prcursors that migrate to the bowel from the neural crest.
- Signals from the migratory and enteric microenvironments determine the fates of the crest-derived ENS precursors.
- Developmental potential is restricted and commitment increases as development proceeds.
  - Stages in development can be recognized by the dependence of cells on a succession of essential transcription factors, growth factors and their receptors.
    - Early factors include Phox2b, Sox10, Ret/GFRa1/GDNF
    - Later factors include Mash-1, EDNRB/EDN3, NT-3/TRkB
- Guidance molecules are needed to colonize the gut and form submucosal and pancreatic plexuses.