Hirschsprung disease is caused by defects in stem cell function

- Neural crest stem cells give rise to the enteric nervous system during fetal development, and persist throughout adult life
- Hirschsprung disease - a failure to form enteric ganglia in the hindgut, leading to potentially fatal gut dysmotility
- Treatment possibilities are limited by incomplete understanding of cause
- We combined gene expression profiling with reverse genetics and analyses of stem cell phenotype and function to uncover links between stem cell function and disease

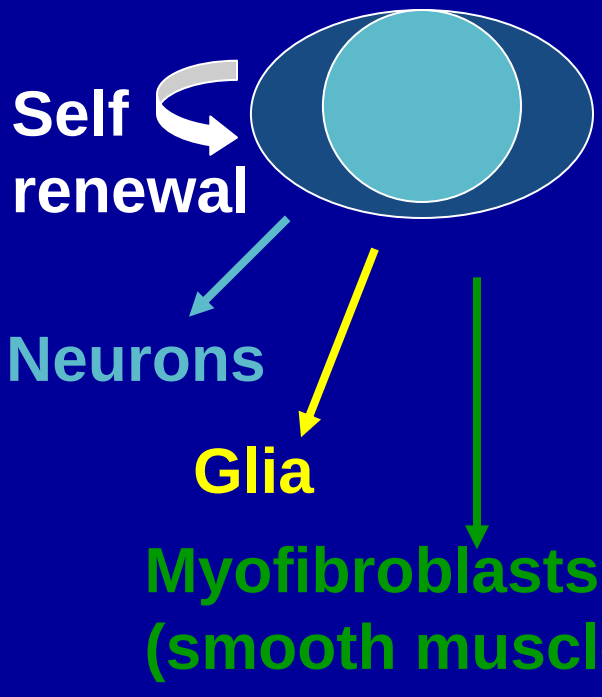
Enteric nervous system (ENS) development

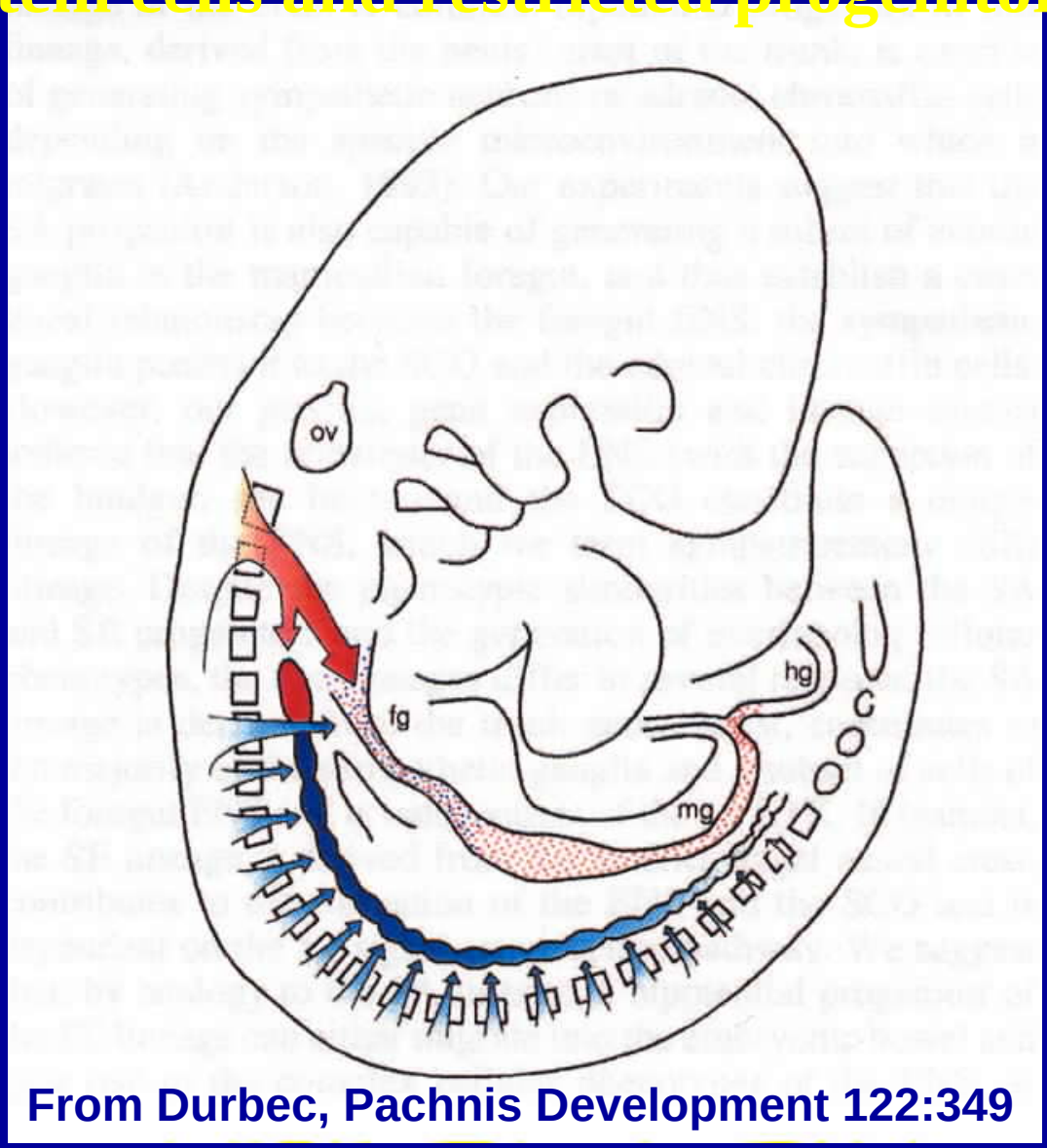
Neural crest includes stem cells and restricted progenitors

Neural crest stem cells

Self-renewing and

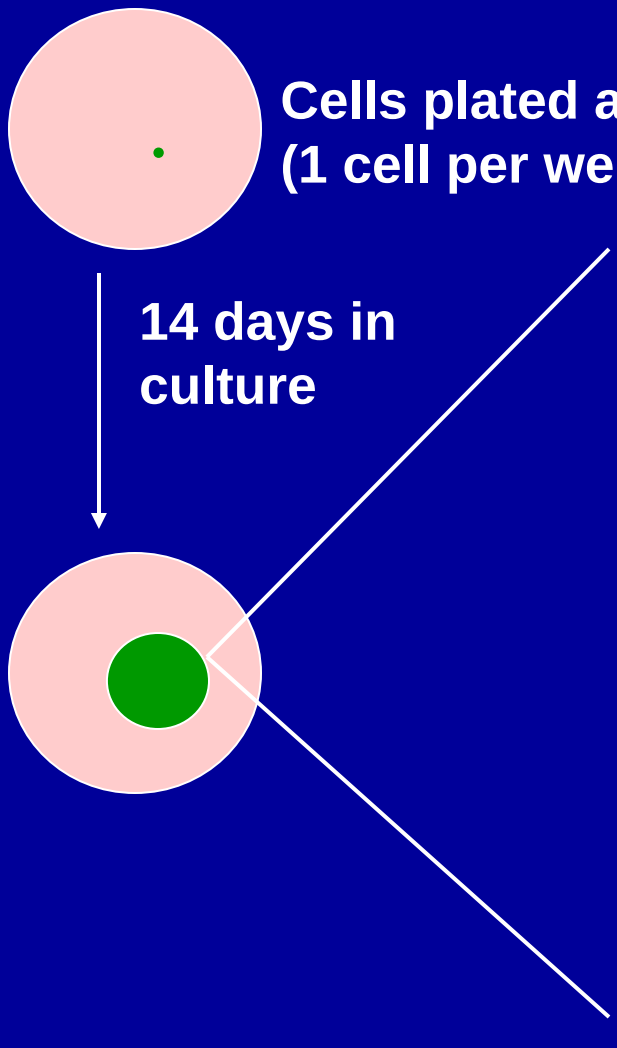
Multipotent





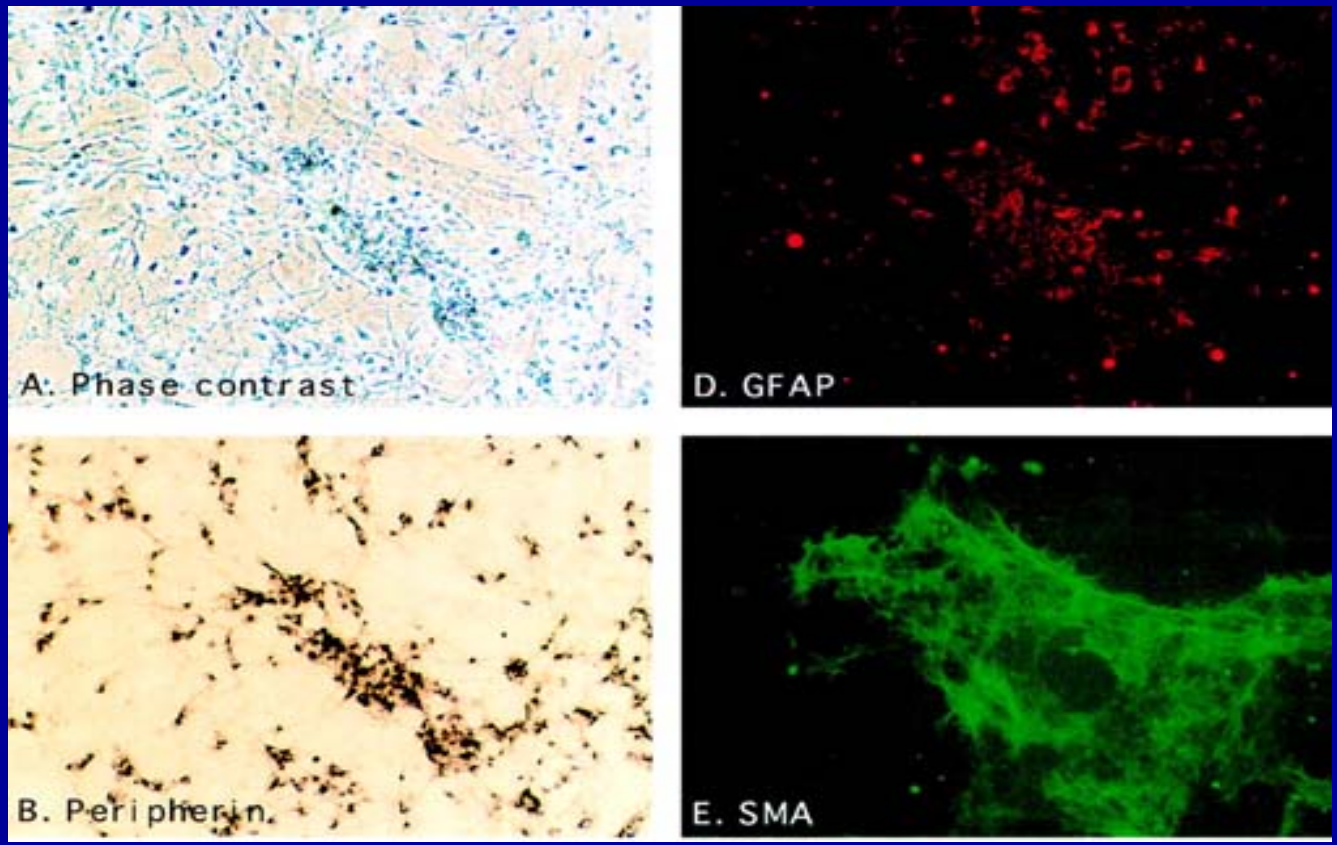
From Durbec, Pachnis Development 122:349

Developmental potential of single cells tested by clonal (single cell) analysis



Cells plated at low density
(1 cell per well, up to 40 cells per 35mm dish)

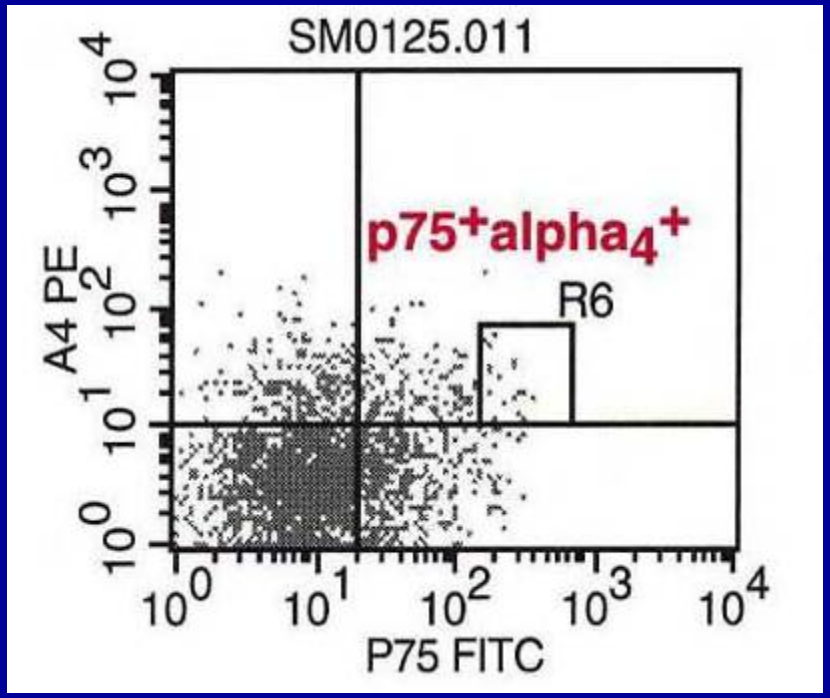
14 days in culture



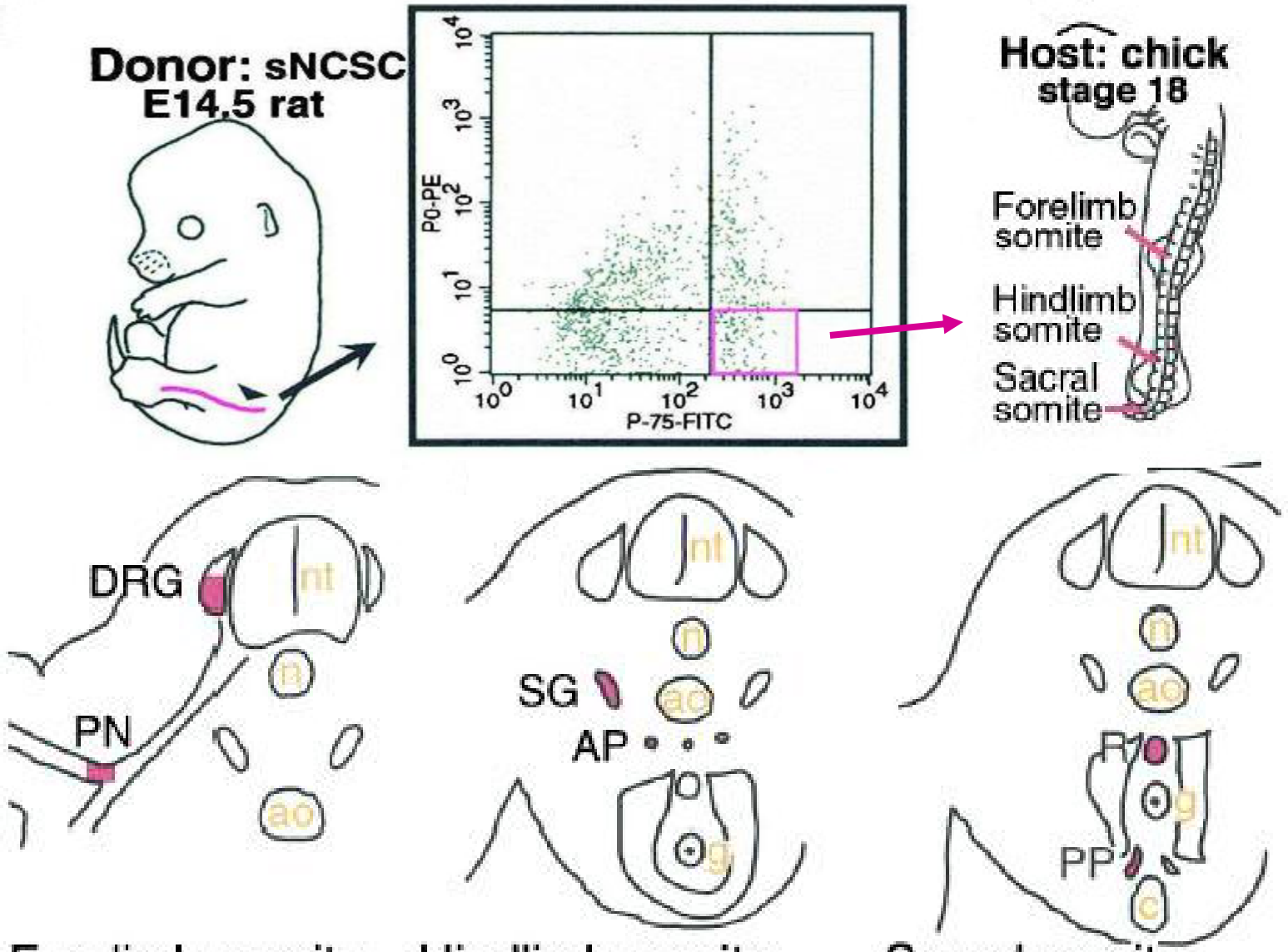
Isolation of fetal gut NCSCs

- **Gut NCSCs give rise to the enteric nervous system during fetal development**

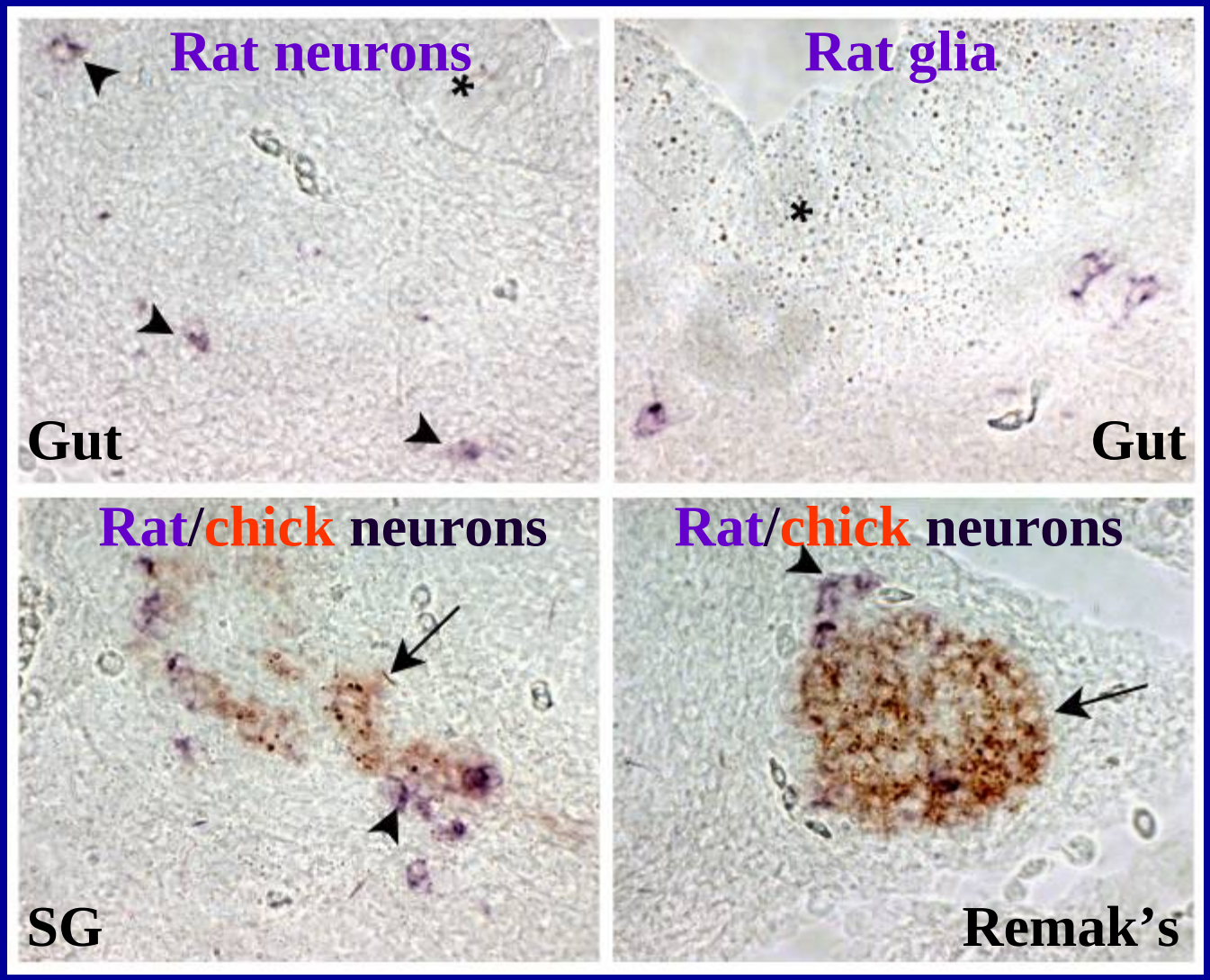
(Neuron 35:643, 2002)



- **Multipotency: 80±7%** of single p75$^+$ α_4^+ cells formed large colonies containing neurons, glia, and smooth muscle cells
- **Self-renewal**: single p75$^+$ α_4^+ cells gave rise to **876±439** multipotent daughter cells after 8 days in culture

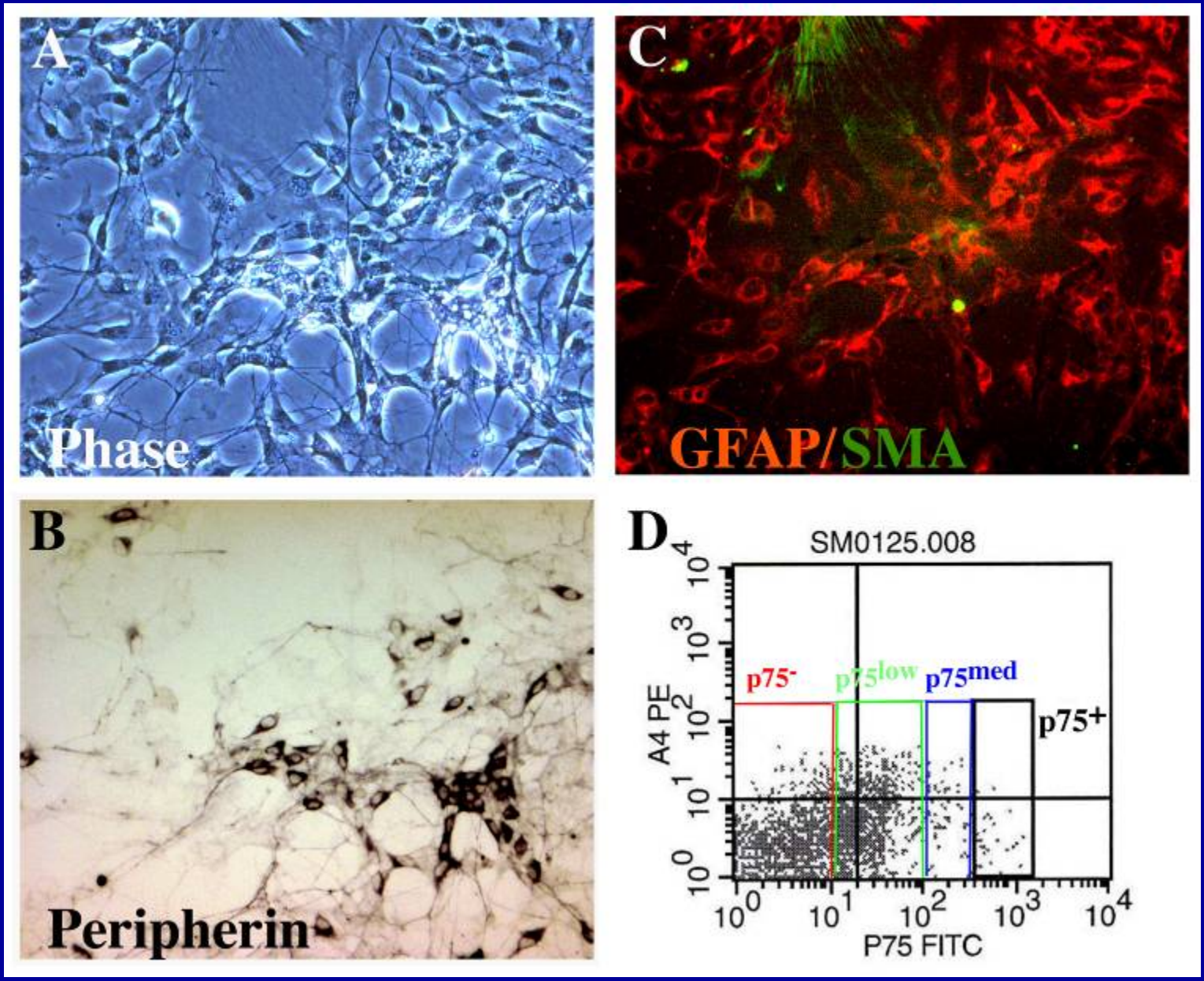

Donor: sNCSC
E14.5 rat
P0-PE
P-75-FITC
Host: chick
stage 18
Forelimb somite
Hindlimb somite
Sacral somite
DRG
nt
n
PN
ao
SG
ac
AP
g
PP
c
Forelimb somite injection
Hindlimb somite injection
Sacral somite injection

Uncultured gut NCSCs give rise to neurons and glia upon transplantation into the developing chick PNS

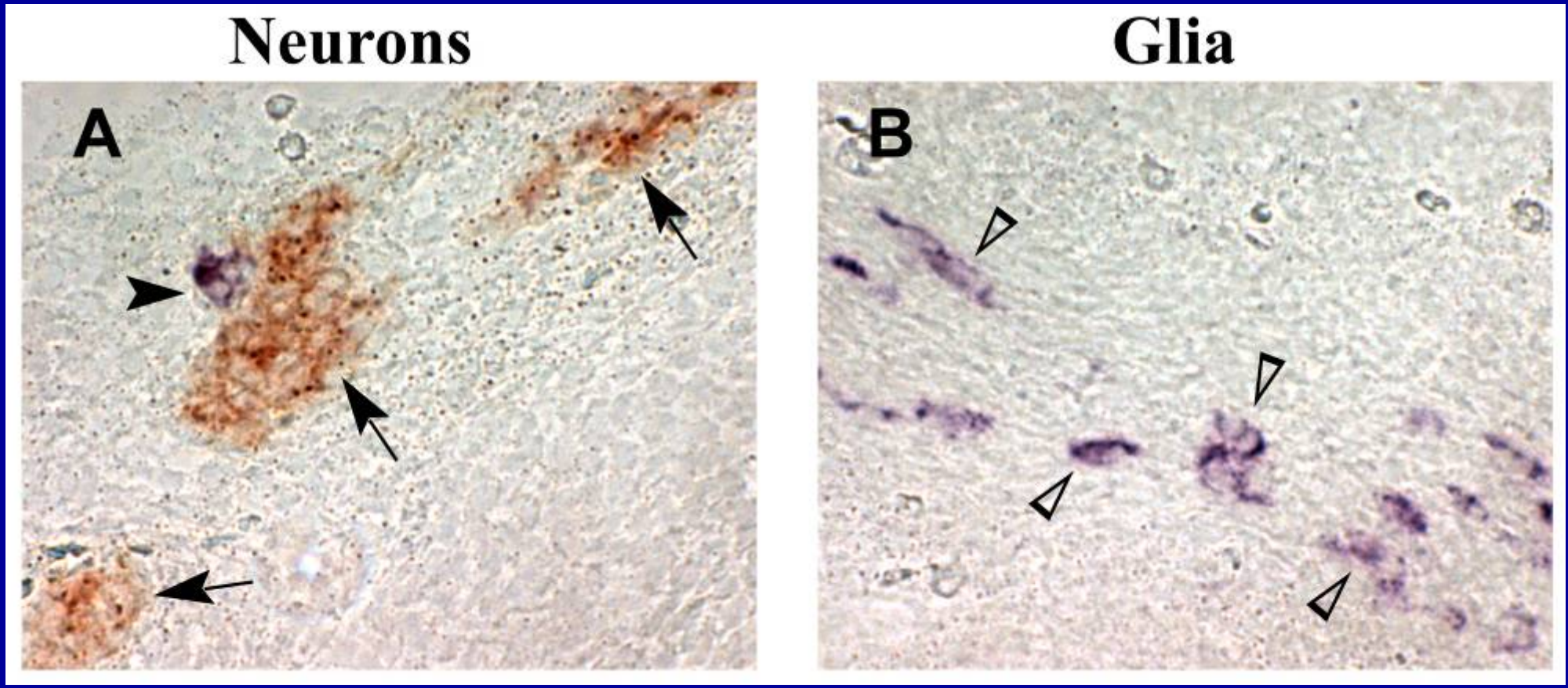


Cell 96:737
Dev. 126:4351
Neuron 35:643

Gut neural crest stem cells persist throughout adult life



Uncultured P15 gut NCSCs give rise to neurons and glia in vivo



Hirschsprung disease is linked to defects in neural crest stem cell function


Toshihide Iwashita


Eve Kruger

the gene expression profile of highly enriched, uncultured neural stem cells

(Science 301:972)

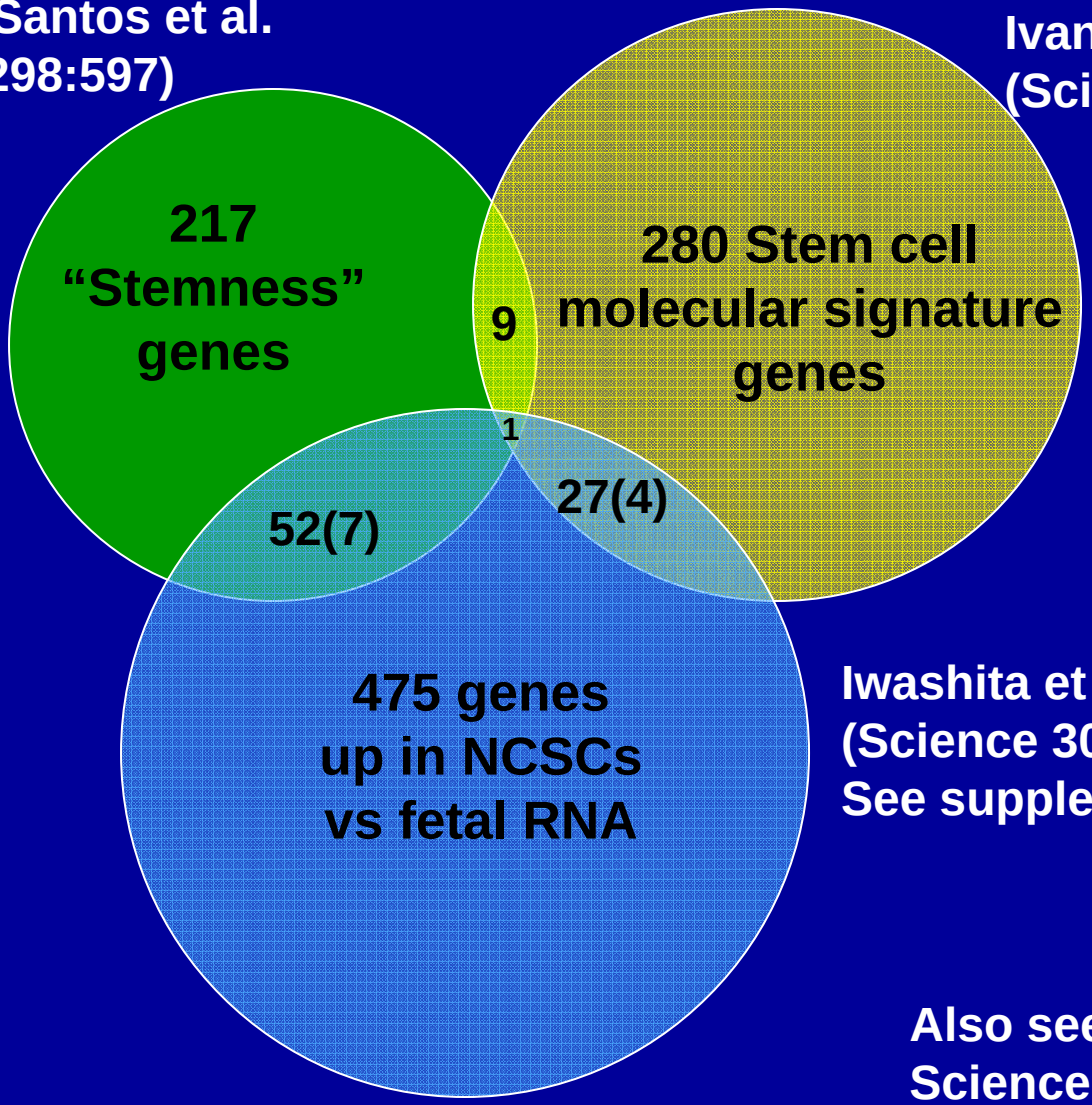
Gene expression profiling of gut neural crest stem cells versus whole fetal RNA

- Affymetrix Rat U34 arrays (26,379 probe sets)
- 3 independent, uncultured aliquots of each cell type
- Differences in expression were confirmed by qRT-PCR in 95% of cases

No evidence for “stemness” genes, or a molecular signature for stem cells

Ramalho-Santos et al.
(Science 298:597)

Ivanova et al.
(Science 298:601)

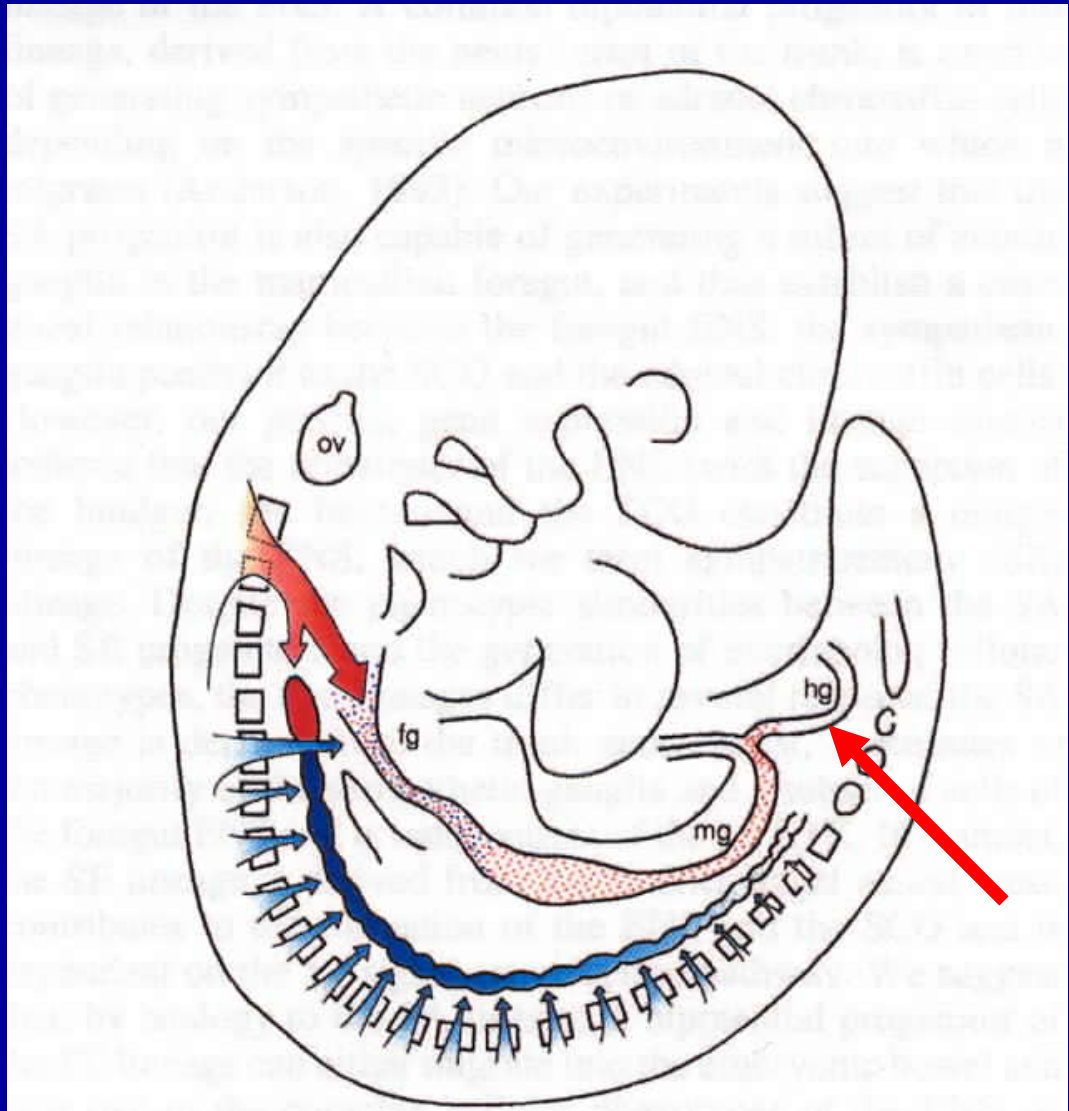


Iwashita et al.
(Science 301:972)
See supplementary table 3

Also see technical comments
Science 302:393

Defects in ENS development: Hirschsprung disease

- Hirschsprung disease is a failure to form enteric ganglia in the hindgut
- mutations have been identified (GDNF/Ret; EDN3/EDNRB)
- mechanism is unclear
- these pathways regulate migration, proliferation, survival and differentiation



From Durbec, Pachnis Development 122:349

4 of the 10 most upregulated genes in gut NCSCs have been linked to Hirschsprung disease

	Unigene Title	Microarray analysis			qPCR
		NCSC	fetus	NCSC/ fetus	NCSC/ fetus
Ret	Ret proto-oncogene	9596	167	57.3	110
DßH	Dopamine ß-hydroxylase	1757	81	17.6	8
CD9	CD9 ANTIGEN	1612	92	16.1	17
ESTs	HS to chromatin structural prot. homolog Supt5hp	1282	15	12.8	
Sox10	SRY-box containing gene 10	1272	23	12.7	17
Gfra1	Glial cell line-derived neurotrophic factor receptor alpha	3846	304	12.6	14
ESTs	Highly Similar to ubiquitin-like 3	1195	74	12.0	
GPRK5	G protein-coupled receptor kinase 5	1175	109	10.8	
Gas7	growth arrest specific 7	3319	309	10.7	
EDNRB	endothelin receptor type B	1159	117	9.9	14

Could Hirschsprung disease be caused by mutations that impair gut NCSC function?

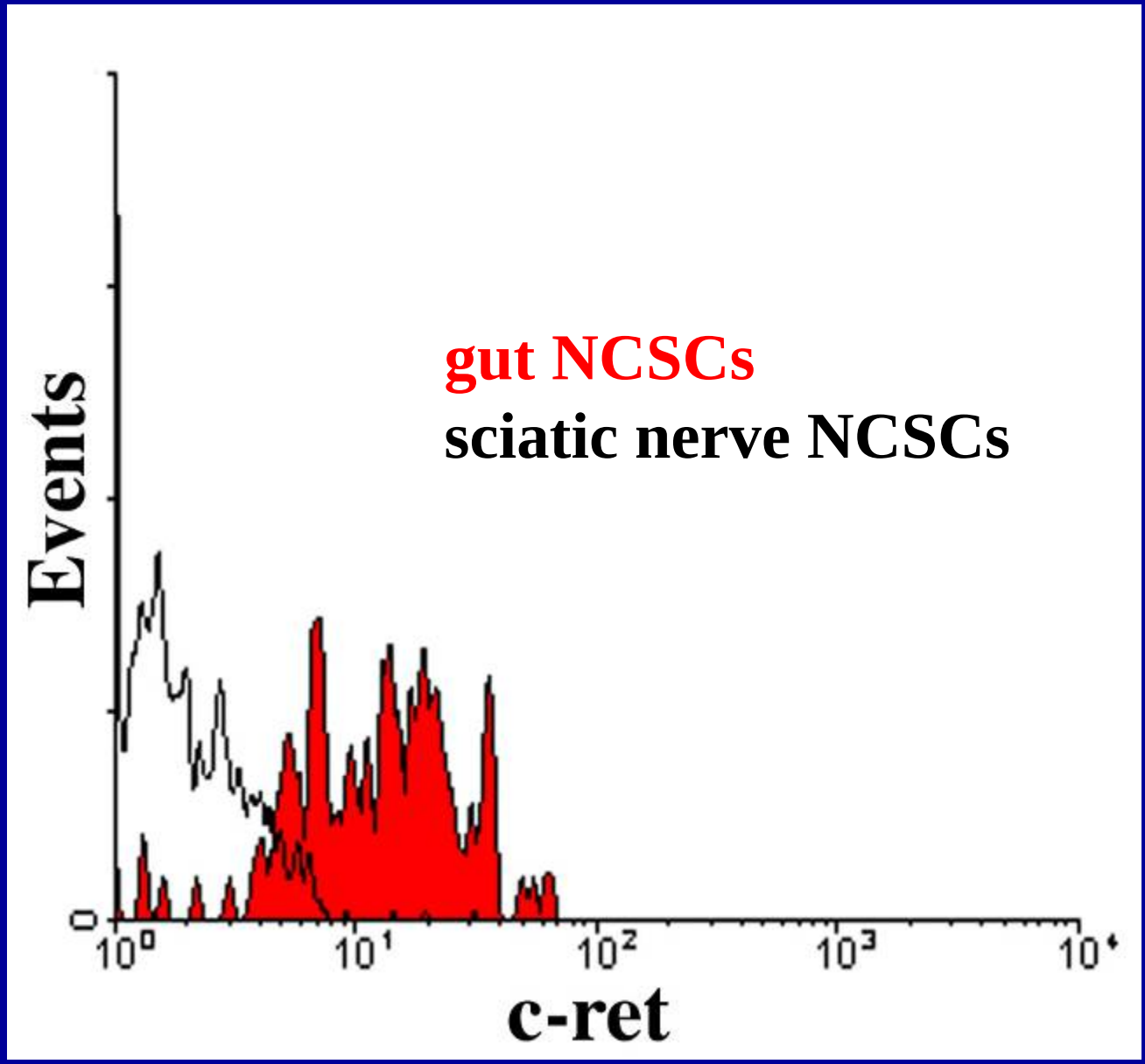
- The cellular mechanism by which the known mutations interfere with ENS development remains uncertain
- It had not been studied whether any of these genes regulate NCSC function
- Many of the mutations that cause or modify the risk of Hirschsprung disease are unidentified (Chakravarti et al., Nat. Genetics 31:89; 32:237)

The GDNF receptor Ret was most differentially expressed between gut NCSCs and whole fetal RNA

	Unigene Title	Microarray analysis			qPCR
		NCSC	fetus	NCSC/ fetus	NCSC/ fetus
Ret	**Ret proto-oncogene**	**9596**	**167**	**57.3**	**110**

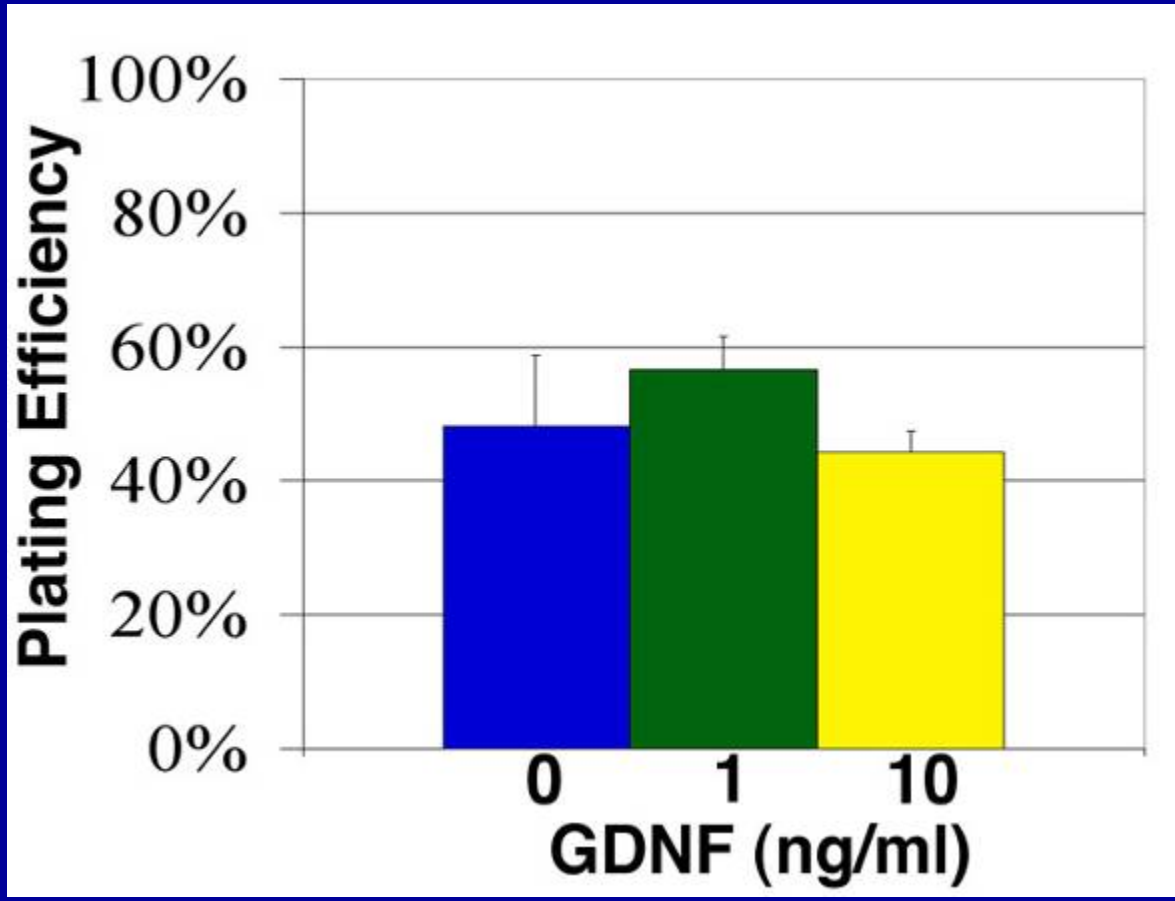
- Without GDNF/Ret, few neural crest cells migrate beyond the esophagus (Pachnis, Rosenthal, Barbacid et al.)
- Mutations in Ret cause Hirschsprung disease in humans and mice

Gut NCSCs express the Ret receptor



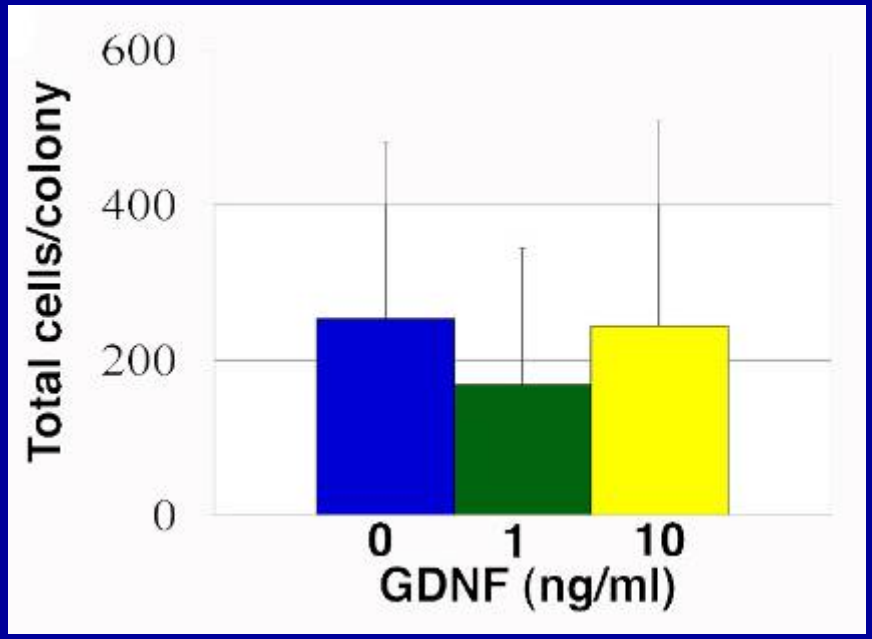
GDNF did not promote NCSC survival in culture

E12.5 or 14.5 rat gut NCSCs were added to culture at clonal density. The addition of GDNF did not increase the fraction of cells that survived to form colonies



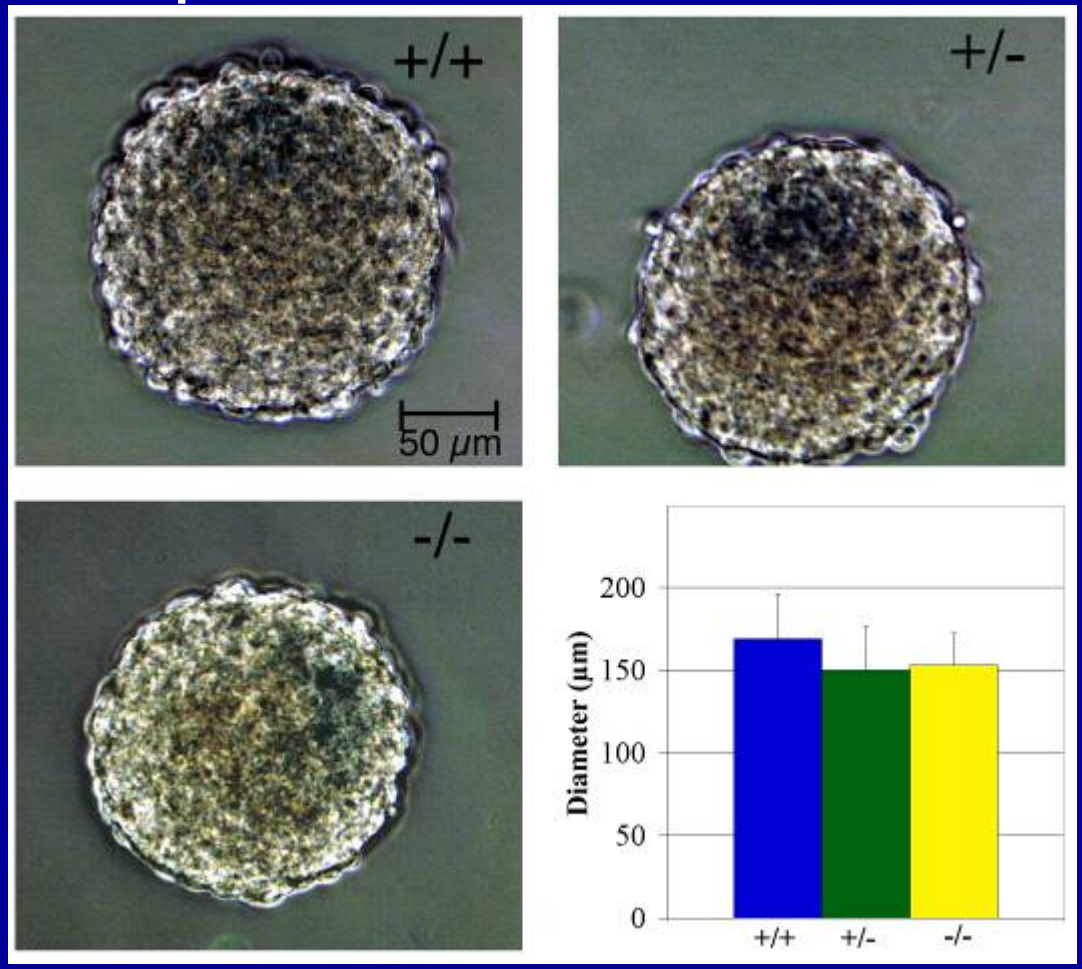
GDNF did not promote NCSC proliferation in culture

GDNF did not affect the proliferation of rat NCSCs over 7 days in culture



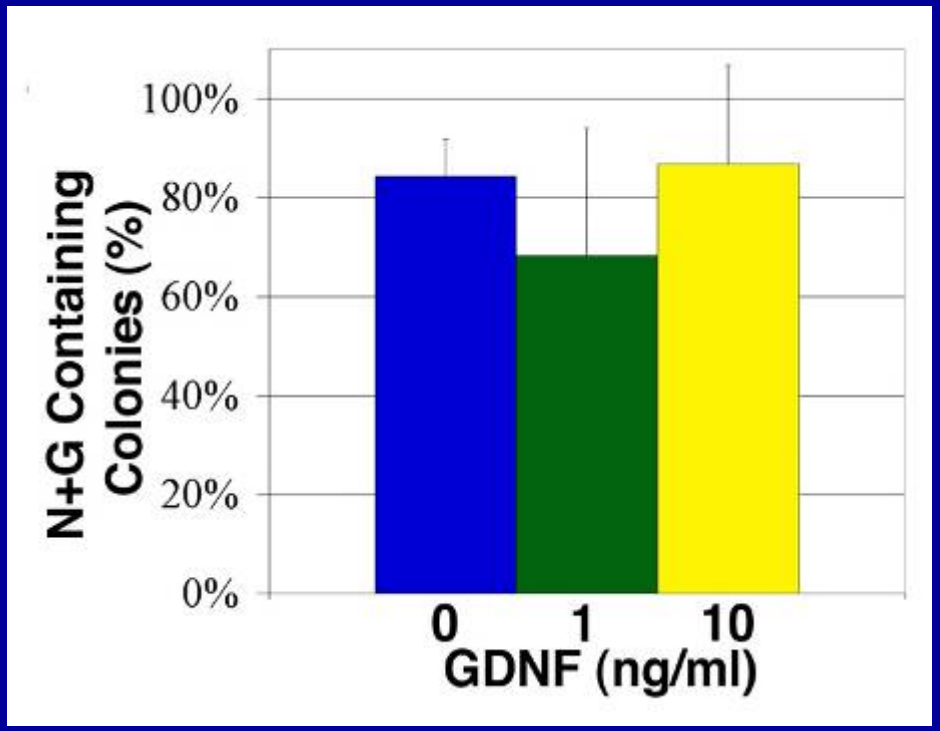
GDNF may promote the proliferation of restricted progenitors (Pachnis et al., Gershon et al., Heukeroth et al.)

Ret deficiency did not affect mouse NCSC proliferation in culture

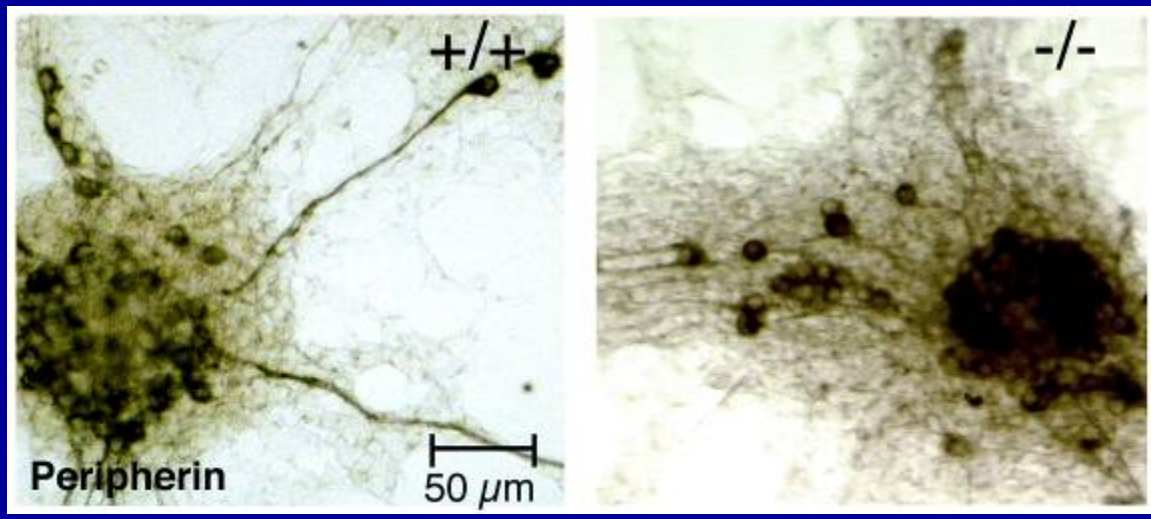


GDNF did not affect the differentiation of NCSCs in culture

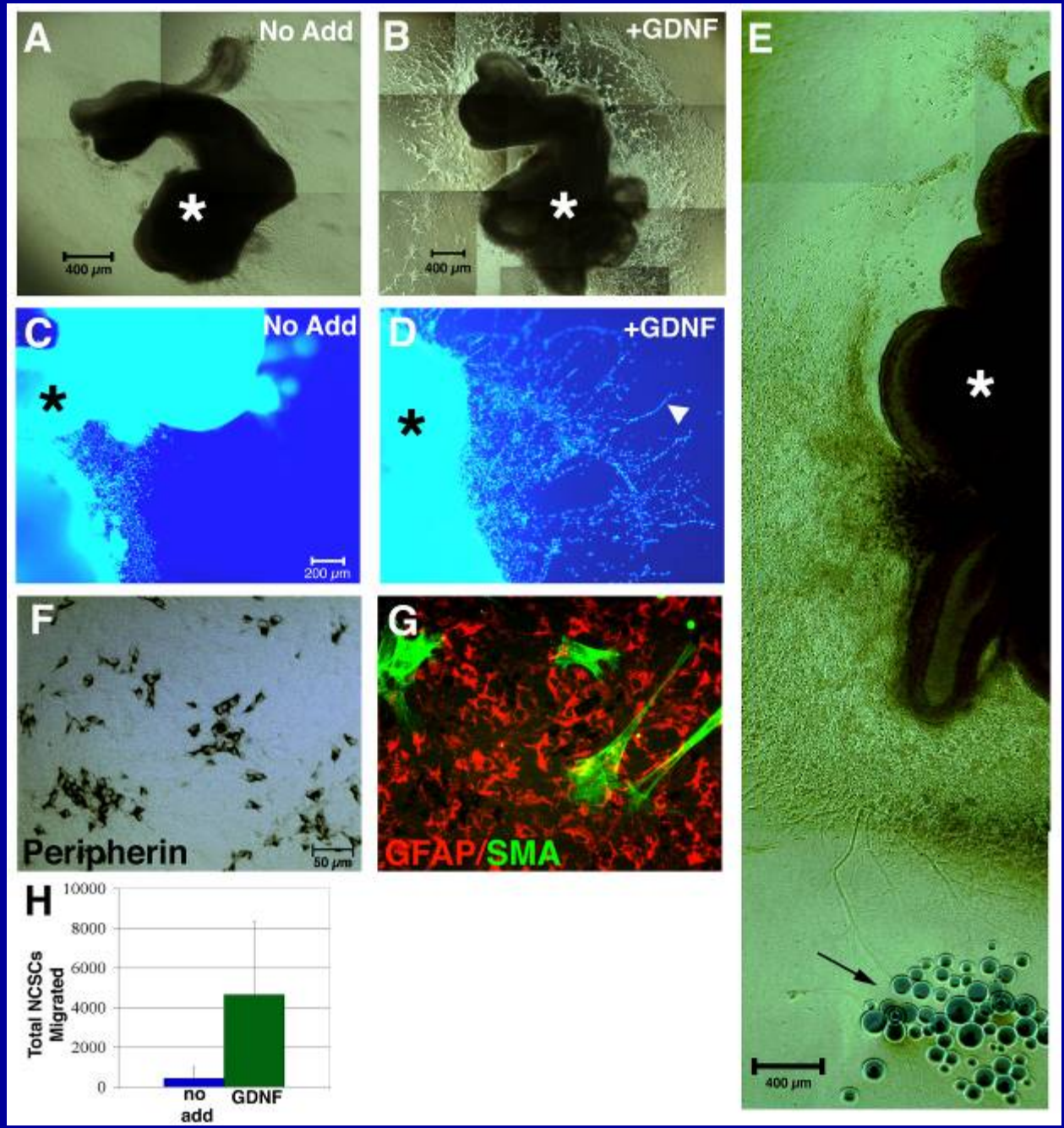
GDNF does not affect the differentiation of rat gut NCSCs in culture



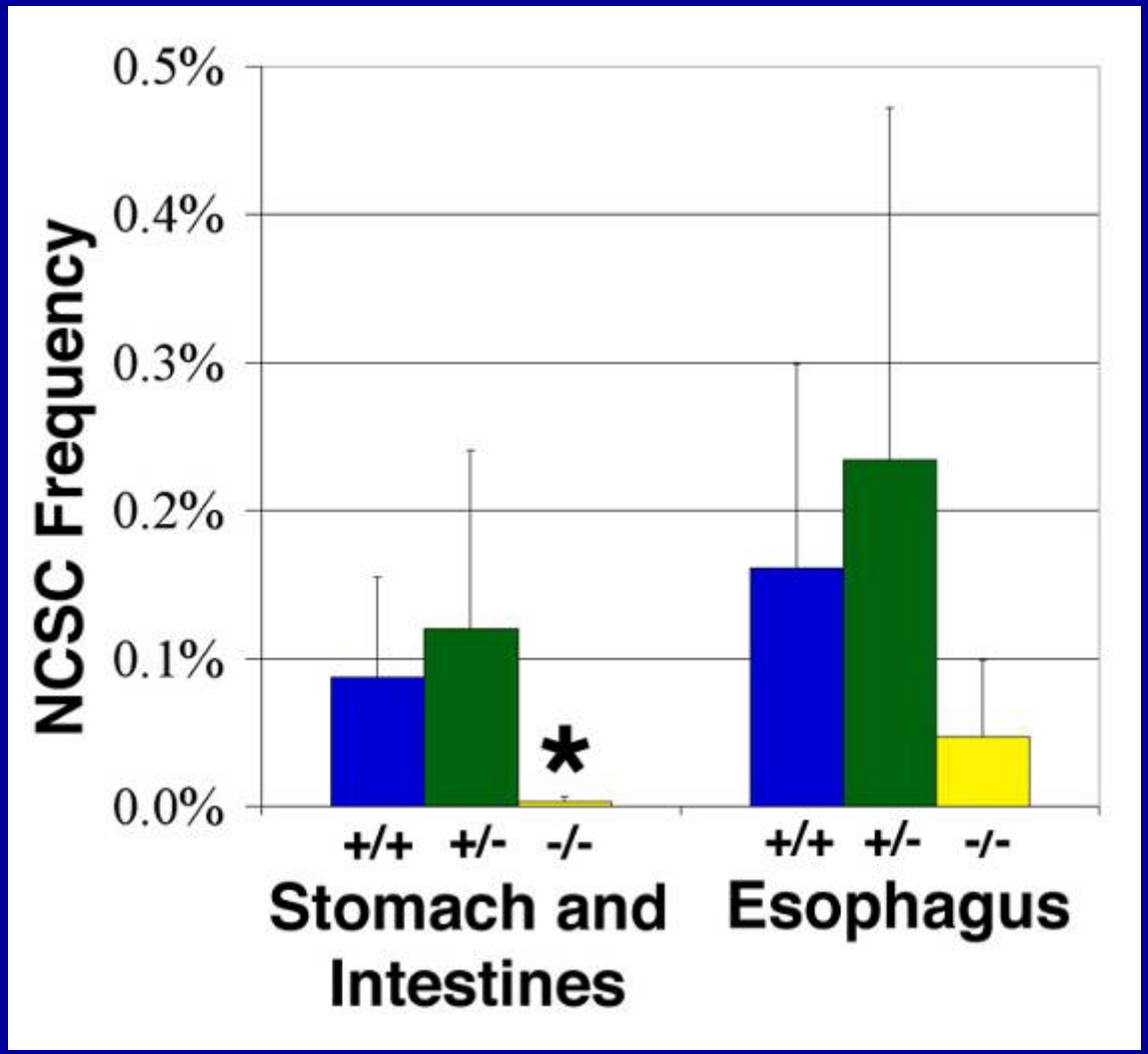
Ret deficiency does not affect mouse NCSC differentiation in culture



- GDNF promotes the migration of NCSCs in culture (Young, Newgreen et al.)
- GDNF is expressed in the gut ahead of migrating neural crest cells in a way that may draw them through the gut (Natarajan, Pachnis Development 129:5151)



Ret (the GDNF receptor) is required for NCSC migration in vivo

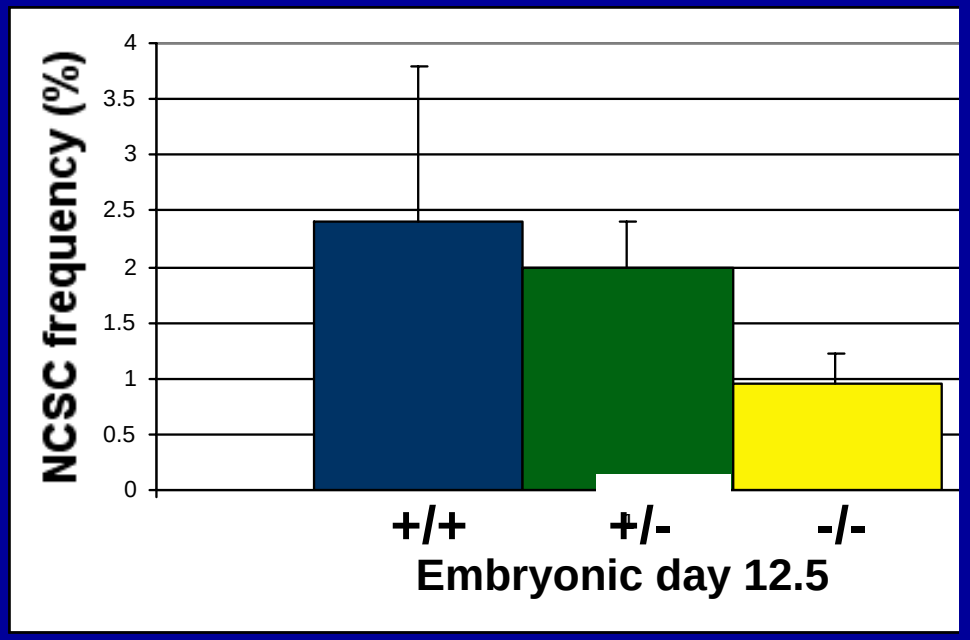


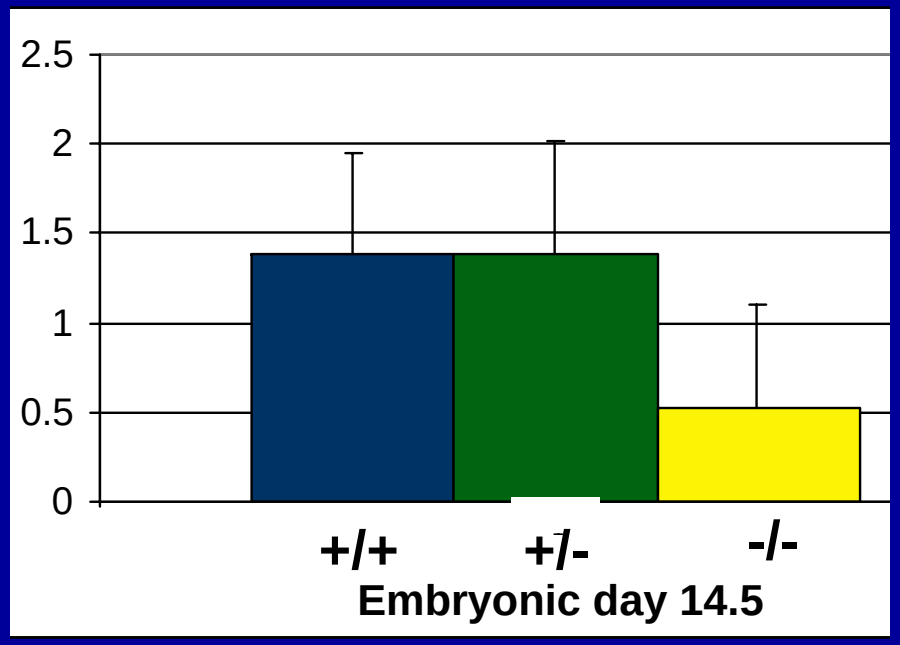
Can we learn more about how different signaling pathways interact to regulate stem cell function and disease risk?

- The endothelin signaling pathway is the other major pathway in which mutations cause Hirschsprung disease
- Does END3/EDNRB signaling regulate NCSC function?
- Can we determine the nature of the interaction between the GDNF and EDN3 signaling pathways at the cellular level?

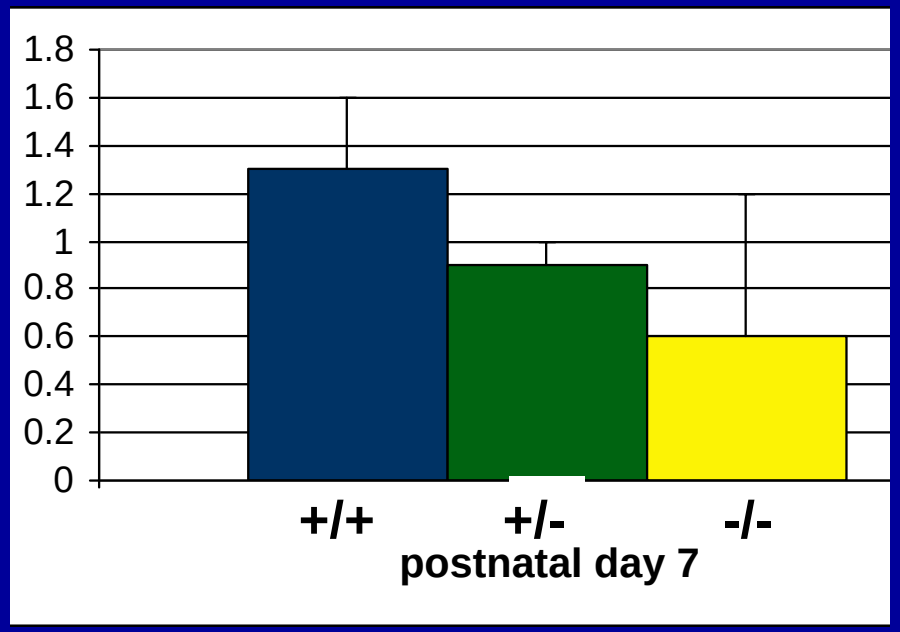
Phenotypically and functionally normal NCSCs are present in the *Ednrb*-/- gut: gut NCSCs do not require Ednrb for their maintenance

	Ednrb	**Multilineage colonies**
$p75^+\alpha_4^+$ cells	**+/+**	**73%**
$p75^+\alpha_4^+$ cells	**+/-**	**86%**
$p75^+\alpha_4^+$ cells	**-/-**	**71%**





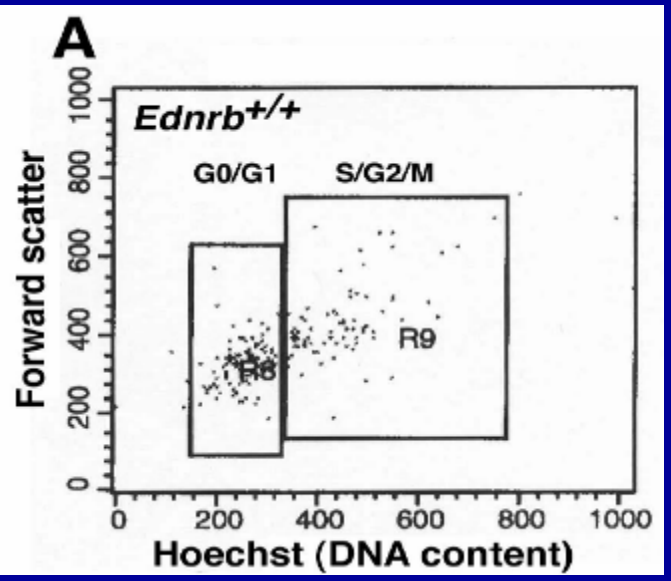
Ednrb is required for the generation of normal numbers of NCSCs in the gut, but not for their maintenance after E12.5

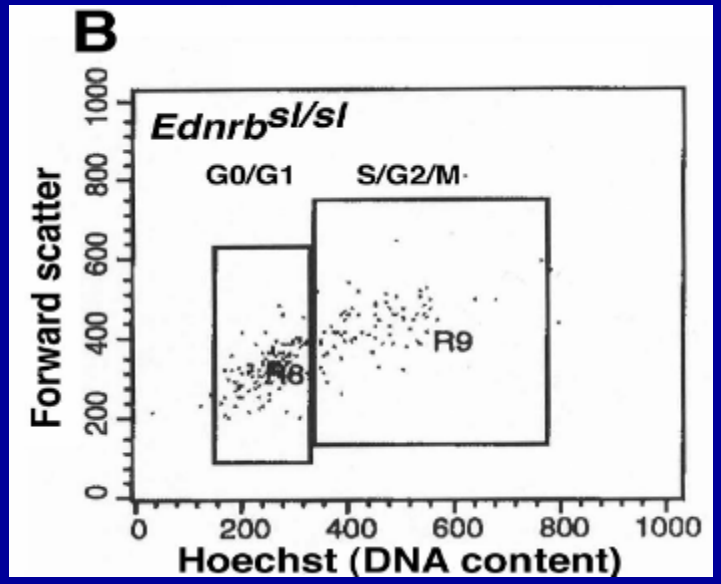


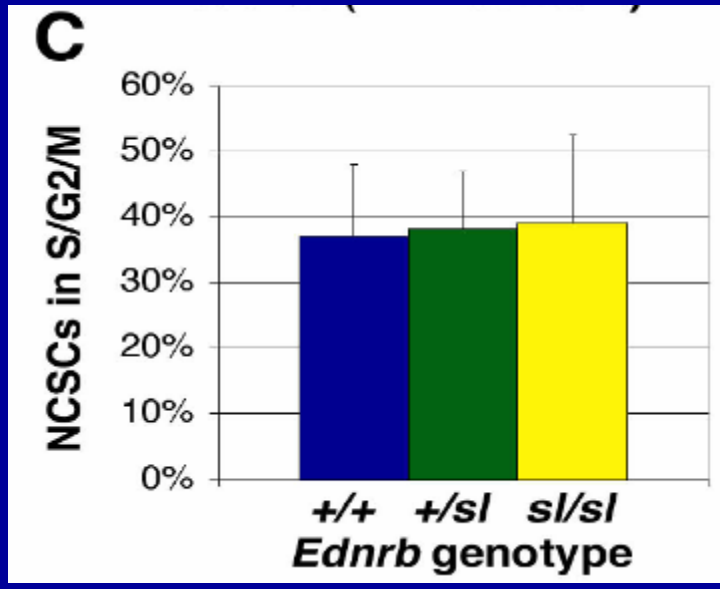
Why does a modest reduction in NCSC frequency lead to complete aganglionosis of the distal gut?

- The literature offers several hypotheses regarding defects in *Ednrb*-/- NCSCs that could impair their ability to colonize the hindgut (E12-E14)
- Perhaps the NCSCs don’t proliferate
- Perhaps they are unable to respond to neurogenic factors
- Perhaps they don’t migrate correctly

Ednrb is not required for the proliferation of NCSCs in vivo at E12.5

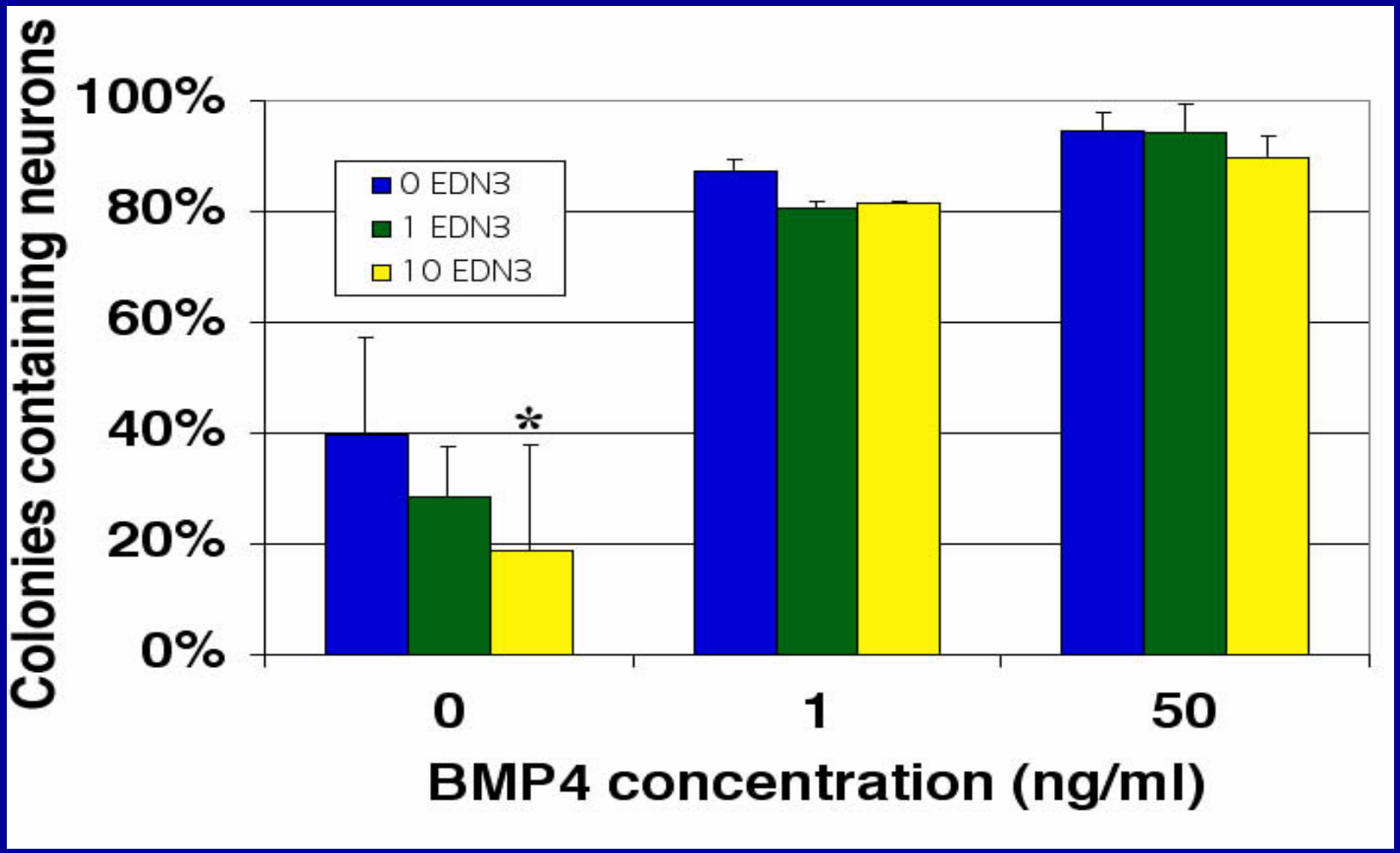




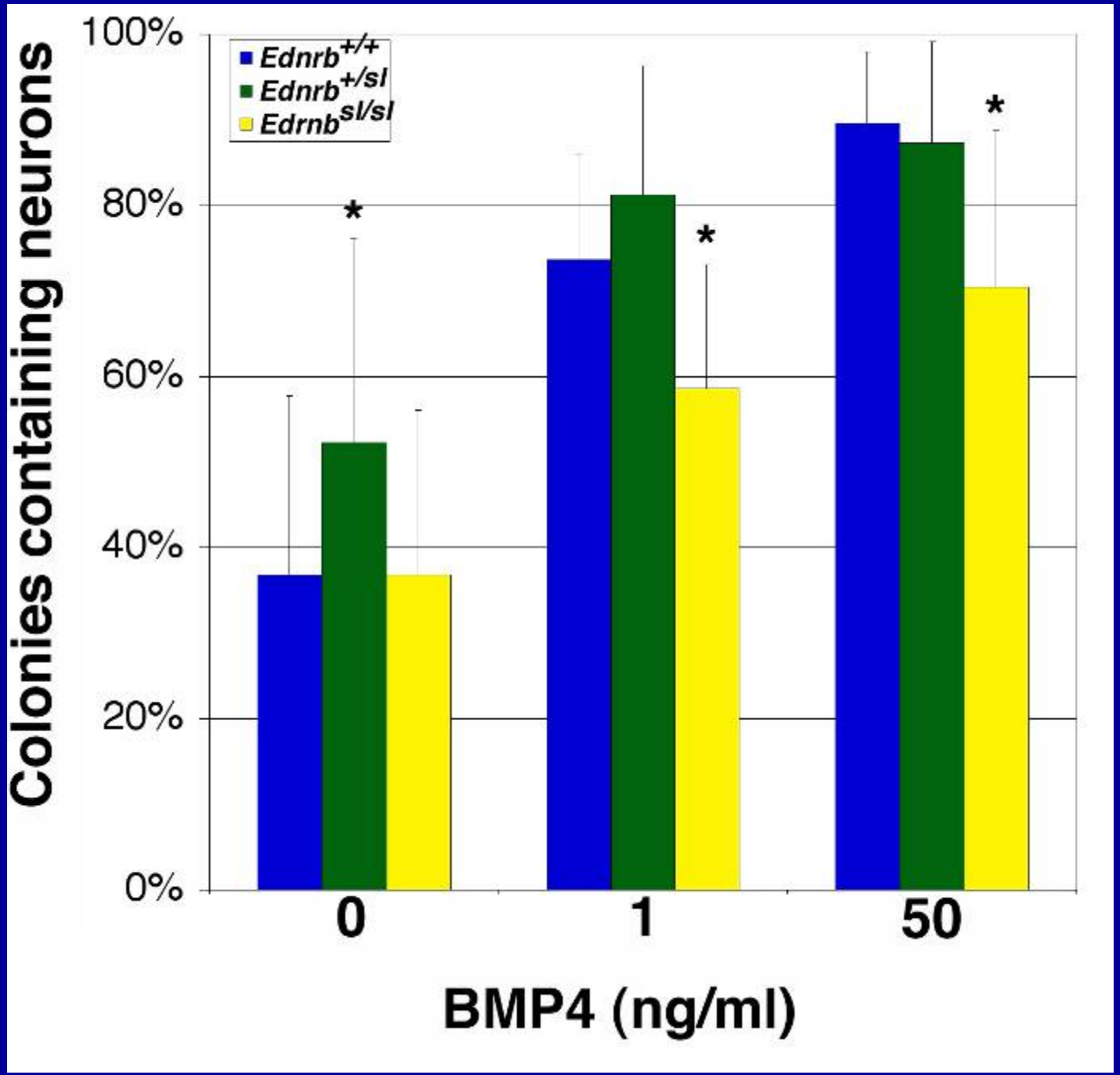


- **It is not clear why Ednrb-/- NCSCs are depleted prior to E12.5, but no further depletion occurs after E12.5, during the onset of distal gut colonization.**

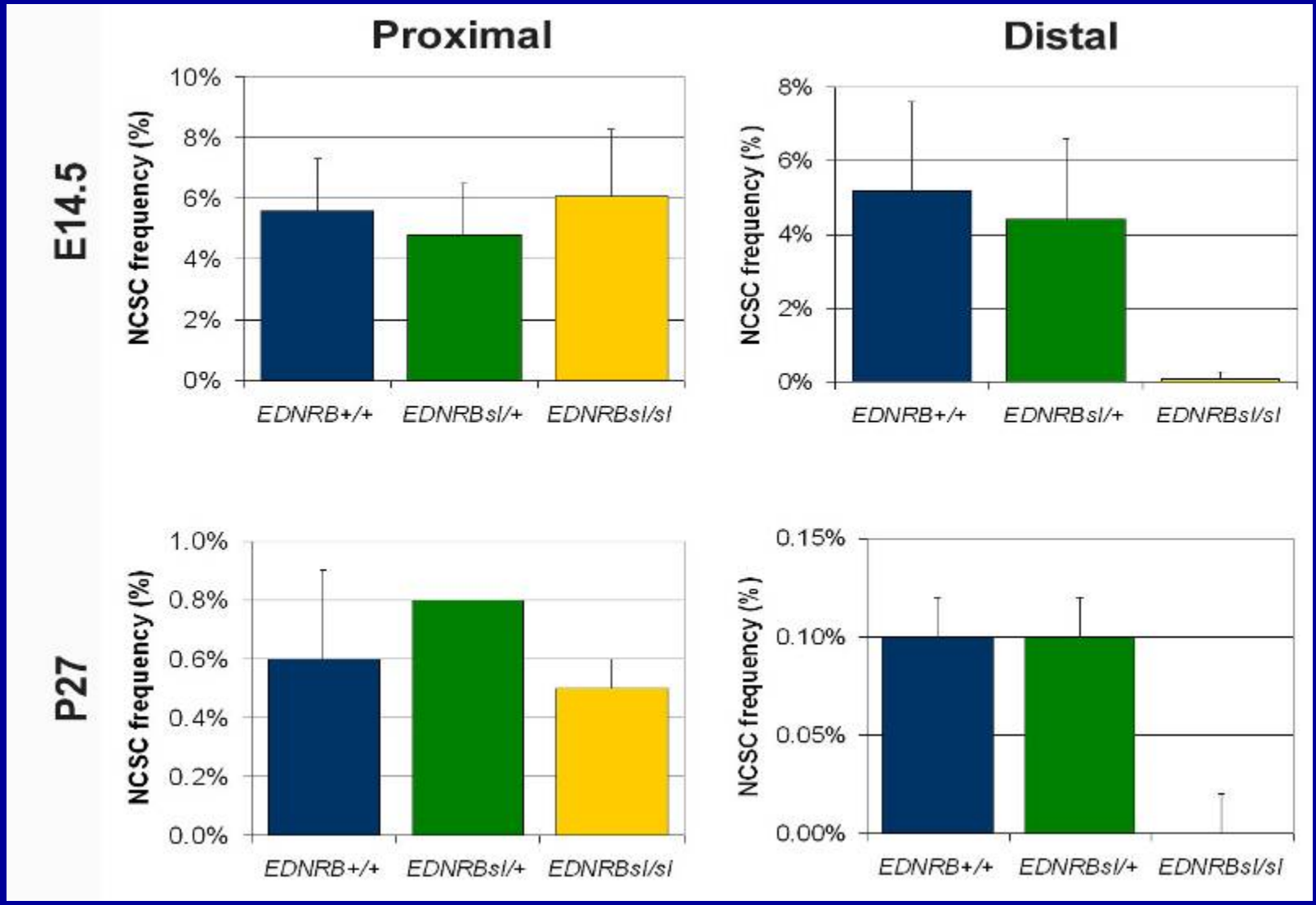
EDN3 does not impair the neurogenic response of NCSCs to BMP4



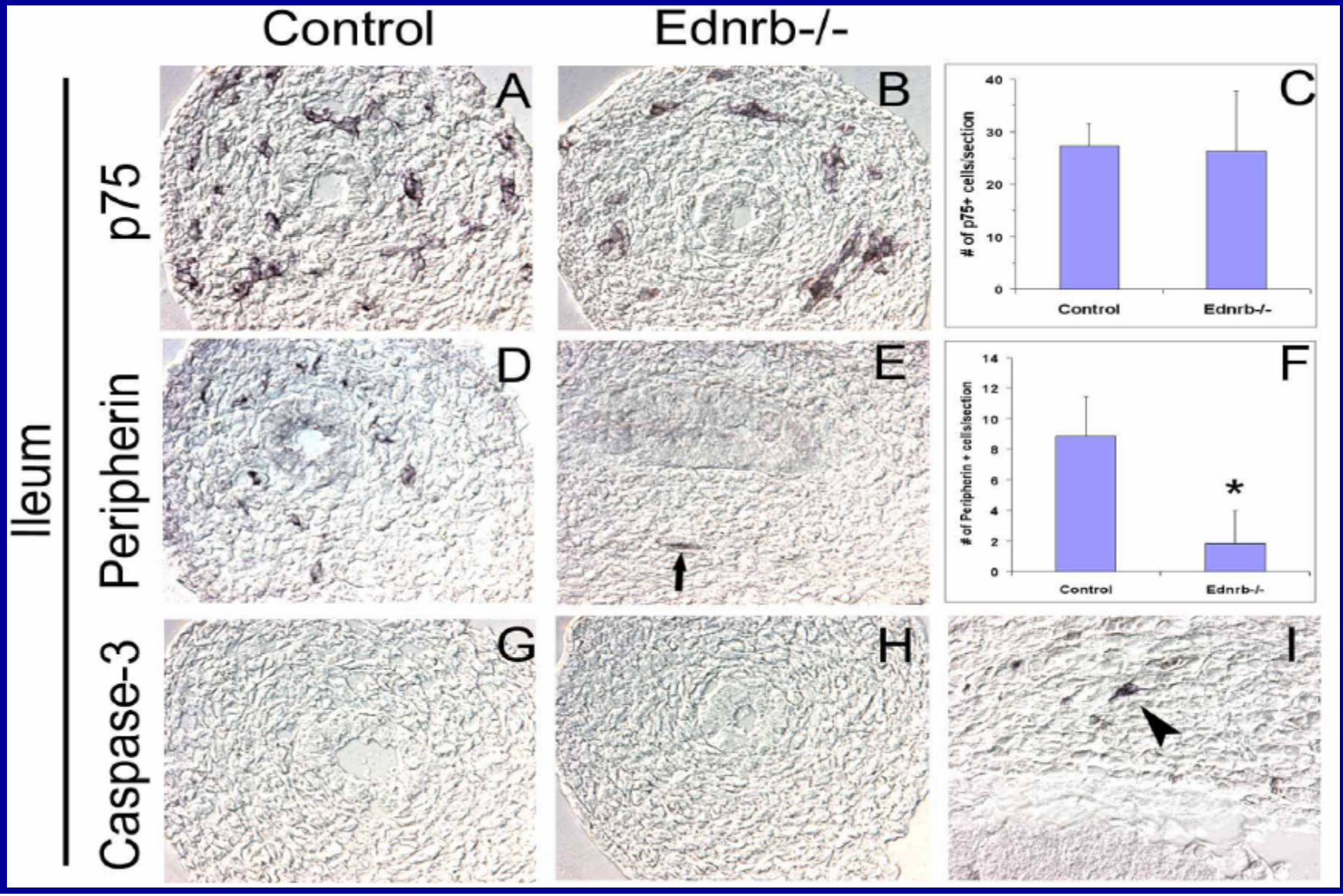
Ednrb-/- NCSCs respond normally to the neurogenic factor BMP4



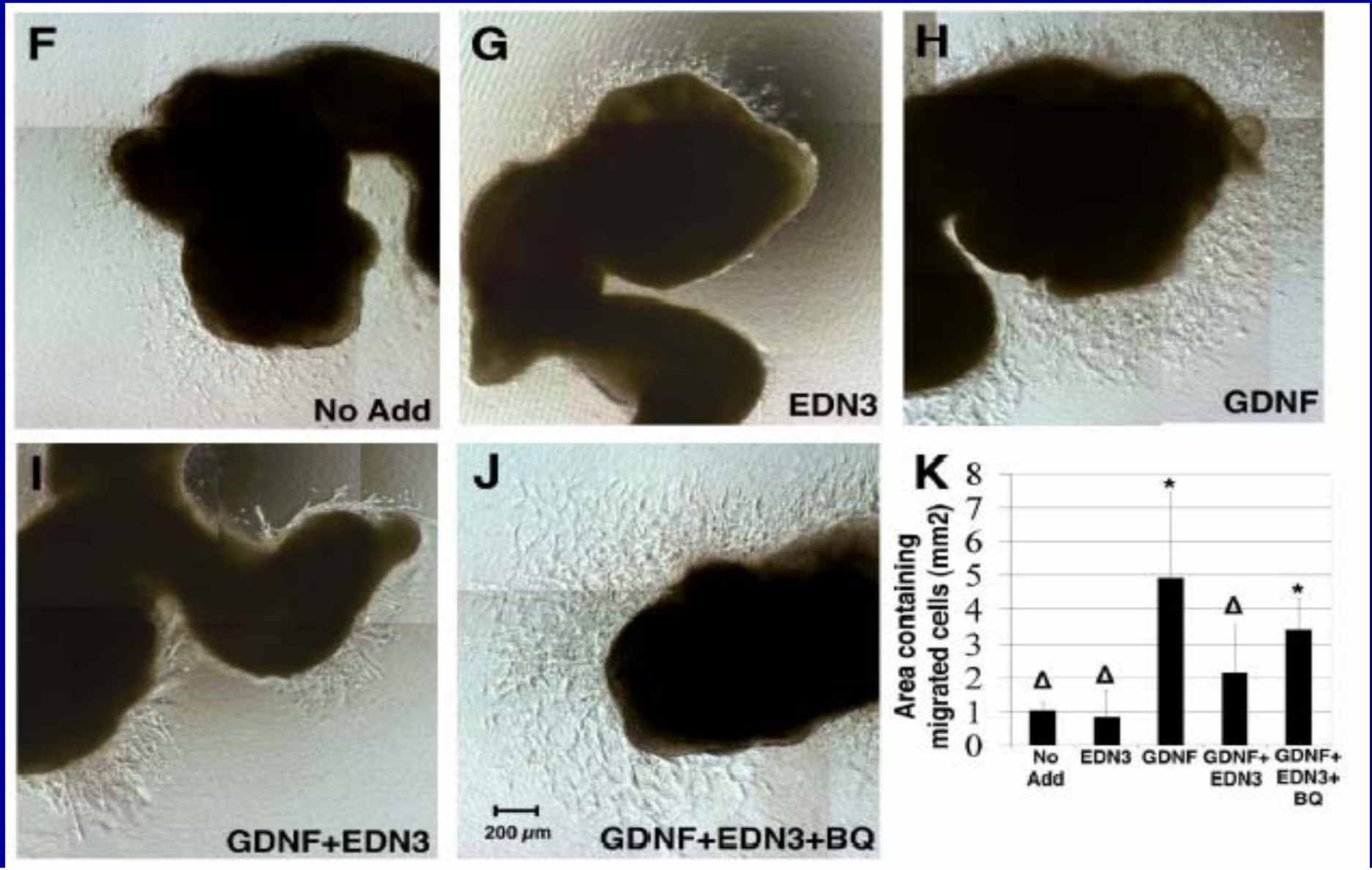
A defect in *Ednrb*-/- NCSC migration: NCSCs never migrate into the distal gut



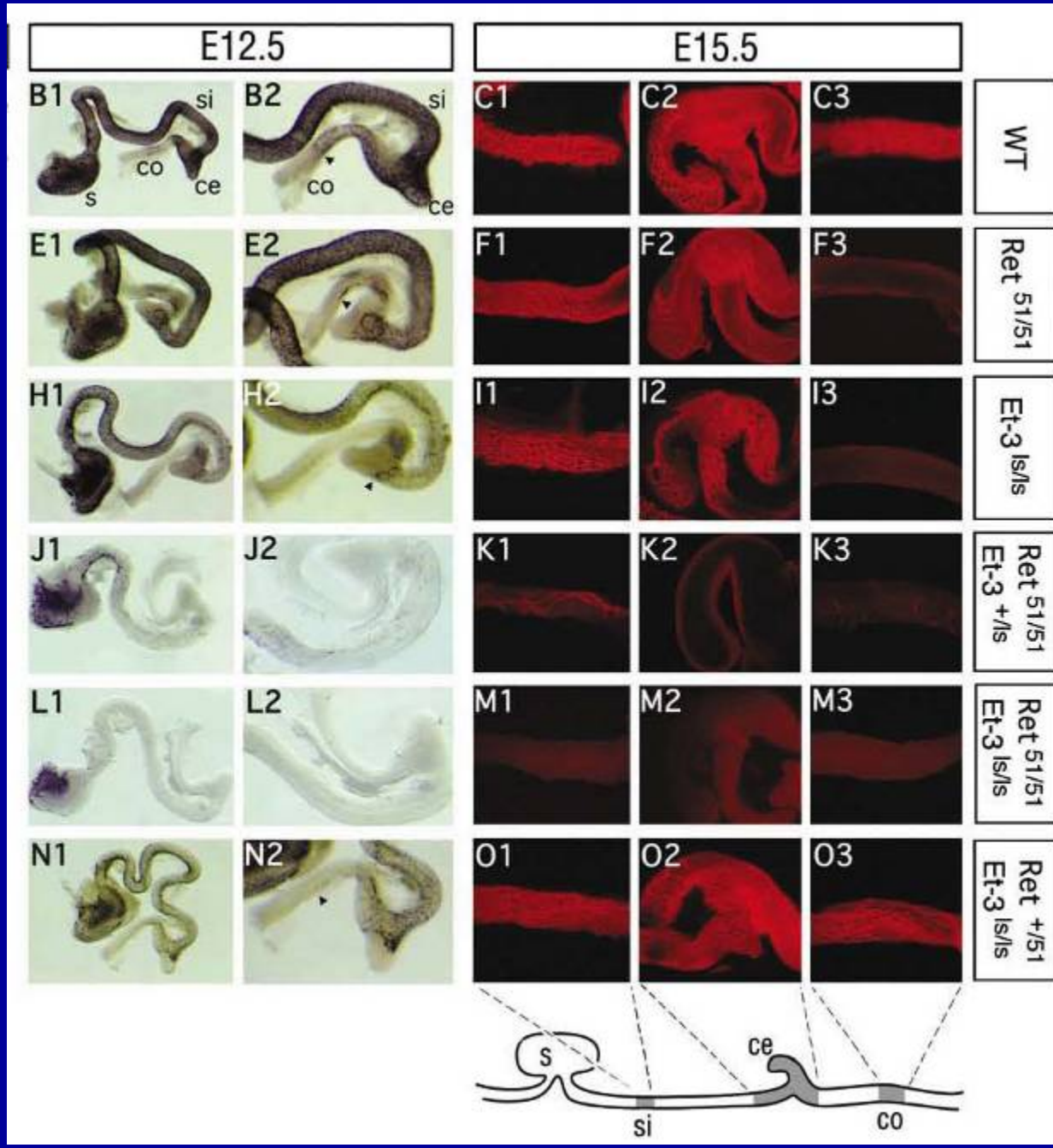
The migration defect is not caused by premature differentiation or cell death at the migration front



EDN3-signaling regulates migration by altering the response to other migratory cues



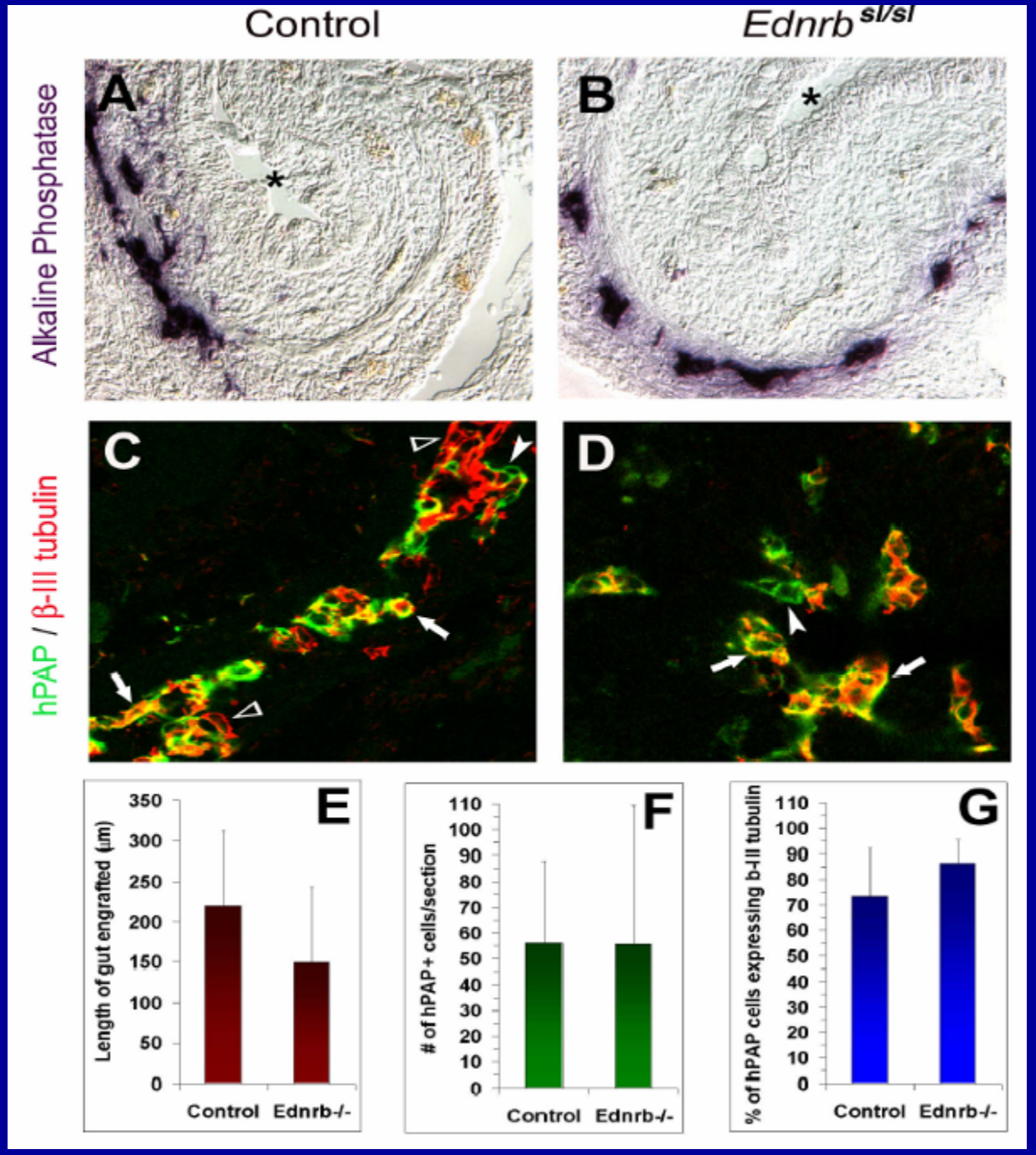
Endothelin signaling in vivo negatively regulates GDNF signaling and interacts to define the size of the progenitor pool (Barlow, Pachnis, 2003)



The EDN3 and GDNF signaling pathways interact to regulate the migration of NCSCs into the distal gut

- This provides a cellular mechanism for the previously observed genetic interaction of these pathways (Chakravarti et al., Nat. Genetics 31:89; 32:237)
- If migration is the primary defect, then can we by-pass this defect by transplanting NCSCs directly into the distal gut?
- Alternatively, is the Ednrb-/- distal gut non-permissive for NCSCs survival or differentiation?

NCSCs survive and form neurons in the *Ednrb*-/- distal gut



Hirschsprung's is caused by defects in the ability of NCSCs to colonize the distal gut

- The GDNF and EDN3 signaling pathways interact to regulate the generation and migration of NCSCs
- These pathways likely have different effects on different subsets of gut neural crest cells
- Now we have a cellular locus in which to study the effects of Hirschsprung mutations: gut NCSCs
- Gene expression profiling may lead to the identification of new mutations that explain human cases
- The prospect of a stem cell therapy for Hirschsprung's

http://www.umich.edu/~stemcell/

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