Getting to the guts of enteric nervous system development

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Summary

Scientists from around the world gathered in New York City recently to discuss the latest research on enteric nervous system development at a meeting organised by Alan Burns and Heather Young. The participants enjoyed 3 days of presentations that spurred active conversations and highlighted the rapidly advancing research in this field.

Introduction

The enteric nervous system (ENS) is a complex network of neurons and glia within the bowel wall that controls many aspects of intestinal function, including motility, epithelial secretion and blood flow. To perform these complex tasks, there are many distinct subtypes of enteric neurons that differ in neurotransmitter expression, morphology, electrophysiology and function (Gershon et al., 1994). The molecular mechanisms that control ENS development were the focus of this meeting.

Although this conference focused mainly on the developmental biology of the ENS, a large fraction of participants were clinicians. This is because developmental defects of the ENS result in Hirschsprung's disease (HSCR), a congenital disorder that is characterised by aganglionosis (the absence of enteric neurons) in the distal colon and affects roughly 1 in 5000 infants (Chakravarti, 2001). This defect causes tonic contraction of the affected bowel segment, resulting in mechanical obstruction. Infants with HSCR often have severe constipation, growth failure and are at risk of dying from the complications of toxic megacolon (Swenson, 2002). The current treatment for HSCR is surgical resection of the aganglionic segment of the bowel, but intestinal dysfunction may persist after surgery. It is presently unclear whether the postoperative morbidity is related to the surgery or to the potentially abnormal function of the residual ENS associated with the primary defect that initially caused aganglionosis. Although absence of enteric neurons is usually restricted to the distal colon, some children have much more extensive aganglionosis, which requires long-term parenteral nutrition for survival. Other less well-understood defects in ENS function include intestinal pseudo-obstruction syndromes and recent evidence indicates that a subset of individuals with irritable bowel syndrome may have primary defects within the ENS. Furthermore, the ENS can be damaged in some forms of chronic disease, especially diabetes. For all of these reasons, understanding the developmental mechanisms that control the migration, survival, proliferation, differentiation and function of ENS precursors and of mature enteric neurons and glia is our best hope of developing novel strategies to diagnose, prevent and treat ENS defects.

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Cellular and molecular mechanisms of ENS development

Colonisation of the gut by neural crest cells

The majority of enteric neurons and glia arise from a small population of cells that originate in the vagal neural crest, invade the foregut and migrate in a rostrocaudal direction through the developing bowel wall (Le Douarin, 1999). Additional crest-derived cells from the sacral region of the neural tube contribute to the post-umbilical ENS and migrate in the opposite direction through the distal bowel (Burns, 2005). To form the mature ENS, precursors must migrate away from the gut entry points and spread uniformly throughout the entire bowel, increase in number and undergo sequential lineage restriction before differentiating into many distinct subtypes of interconnected neurons and glia. The end result of all this is the formation of an integrated neuronal network within the myenteric and submucosal plexi (Grundy and Schemann, 2005) (Fig. 1). Because of the considerable length of the gut and the relatively long period required for its colonisation by ENS progenitors, subpopulations of enteric neural crest cells, at a given moment and place along the gut, face different challenges and have different priorities. The main challenge in the field of ENS development is to identify the molecular signals that control the migration, survival, proliferation, differentiation and connectivity of ENS progenitors and enteric neurons and to understand the mechanisms that co-ordinate these cellular processes in time and in space. Given the complexity of the ENS, it is not surprising that many molecules have already been implicated in its development and organisation, and many more are waiting to be discovered.

Previous reports have suggested that ENS precursors migrate along the bowel primarily in response to the chemoattractant effect of glial cell line-derived neurotrophic factor (GDNF) (Natarajan et al., 2002; Young et al., 2001). Although this view is consistent with the phenotype of RET-, GFRα1- and GDNF-deficient mice, it is based solely on in vitro assays, and to date no clear evidence is available to prove an in vivo role for the GDNF/RET signalling pathway in enteric neural crest cell migration. Moreover, two papers presented at this meeting challenged the idea that GDNF is the driving force for the rostrocaudal migration of neural crest cells, and put forward the view that proliferation is the main factor that sustains migration. A combination of mathematical modelling and organ culture experiments suggest that 'population pressure' resulting from proliferation alone (and in the absence of chemotactic signals) could drive ENS precursor migration down the length of the bowel (Don Newgreen, Royal Children's Hospital, Melbourne, Australia). Consistent with these findings, Alan Burns (Institute of Child Health, London, UK) showed in work performed with Amanda Barlow (Institute of Child Health, London, UK) that ablation of neural crest at the level of somites (S)3-6 reduced the number of ENS precursors in the bowel and resulted in distal aganglionosis. Assuming that the main effect of the ablation is a reduction in the number of ENS progenitors that invade the foregut, these findings highlight the requirement for a critical number of ENS progenitors for the normal colonisation of the bowel, and indicate that vagal neural crest cells derived from levels S1, S2 and S7 have limited capacity to compensate for the ablated segments. This analysis does not, however, eliminate the possibility that proliferating ENS precursors compete for limited local sources of GDNF, thus creating gradients that, in turn, have chemotactic effects on migrating ENS precursors. The caveat of multiple interpretations notwithstanding, these reports
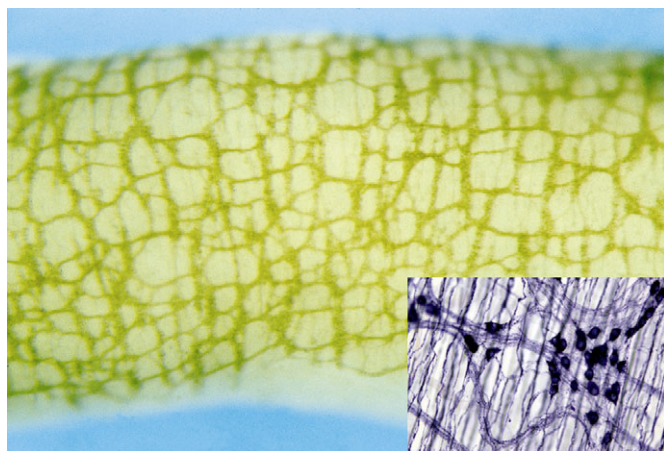
Fig. 1. Acetylcholinesteratse histochemistry on a whole-mount preparation of newborn mouse gut highlights the network of enteric ganglia. A detail of the ENS plexus (stained for NADPH diaphorase) is shown in the inset. Image courtesy of Esther de Graaff (Erasmus University, Rotterdam, The Netherlands) and Robert O. Heukerch.

make a significant conceptual advance, in that they ask us to look at the migrating ENS precursors as a population with properties that do not simply represent the sum of the individual cells.

Using more traditional assays, many additional molecules have now been implicated in controlling specific aspects of ENS precursor migration, including BMP4 (as presented by Alcmène Chalazonitis, Columbia University, NY, USA; Ming Fu and Robert Heuckeroth, Washington University School of Medicine, St Louis, MO, USA), endothelin 3 (as presented by Allan Goldstein, Harvard Medical School, Boston, MA, USA), sonic hedgehog (as presented by Vincent Lui, University of Hong Kong, Hong Kong), L1CAM (as presented by Richard Anderson and Heather Young, University of Melbourne, Melbourne, Australia), ephrin B1 (as presented by Carol Erickson, University of California, Davis, USA), netrin and DCC (as presented by Elyanne Ratcliffe and Michael Gershon, Columbia University, New York, NY, USA), and PKCζ (as presented by Bhupinder Vohra and Robert Heuckeroth, Washington University School of Medicine, St Louis, MO, USA).

New players in ENS development

Despite significant progress having been made in recent years, many aspects of ENS development remain poorly understood. For example, the mechanisms that control ENS precursor differentiation into specific neuron subtypes or that regulate patterns of neurite extension are largely unknown. The trophic factors that support subsets of mature enteric neurons have also yet to be completely evaluated. Even when all of the molecules currently known to control specific aspects of ENS development are considered, the complexity of the ENS cannot be adequately explained by the available molecular tools. Two presentations by Robert Heuckeroth and Tiffany Heanue (National Institute of Medical Research, London, UK) provided new data that were generated by comparing gene expression patterns in the gut of wild-type and RET-deficient mice, which are aganglionic. These studies led to the identification of numerous genes, many of which were not previously known to be expressed in the ENS. Among these newly identified genes are those that probably function in the migration of ENS progenitors, in axon outgrowth and pathfinding, in synaptic function, in vesicle trafficking, and in transcriptional regulation.

Genetic manipulations in mice and zebrafish, together with the ability to modulate gene expression in cultured ENS progenitors and in enteric neurons, promise to uncover the specific roles of many of these genes over the next few years. The power of this genome-wide approach transcends the compilation of more or less complete lists of genes important for ENS cell function and has implications for medical genetics, as human homologues of genes identified in these screens map to previously identified HSCR susceptibility loci. The potential role of such genes as modifiers of the HSCR phenotype remains to be established. The importance of identifying new candidate genes for HSCR in medical genetics was further highlighted by the recent identification of *KIAA1279*, which is mutated in Goldberg-Shprintzen megacolon syndrome (Alice Brooks, ErasmusMC, Rotterdam, The Netherlands; Jean-Marie Delalande, Emory University, Atlanta, GA, USA). Finally, novel and exciting proteomic approaches to identify molecules expressed in the ENS were also presented (Cornelia Hagl, University of Heidelberg, Mannheim, Germany).

Although much of our current understanding of ENS development and HSCR is based on experiments with chick embryos (an ideal system for manipulations of the neural crest) and on mouse and human genetics, it became clear at this meeting that other vertebrate model organisms, such as the zebrafish and the frog, offer new opportunities for the field of ENS development, which stem from the ability to combine powerful genetics (as in zebrafish) with embryonic gene expression manipulation and the live imaging of fluorescently tagged (ENS) progenitor cells. Using real-time video microscopy, it is now possible to examine the motility of the zebrafish gut within the intact organism, a task that in other species can be achieved only in short-term organotypic cultures of intestinal segments. At this meeting, we were given only a relatively small taste of the exciting new field of zebrafish ENS biology. Two speakers (Judith Eisen and Julie Kuhlman, University of Oregon, OR, USA) reported forward genetic screens and the identification of several loci that control various aspects of vagal neural crest biology, from their origin in the CNS to their arrival within the gut. The ability to examine in detail the peristaltic activity of the gut in these mutants offers the exciting opportunity to correlate directly the underlying neuronal deficit and the resulting dysmotility. One of the mutant loci (*lessen*, *lsn*) (discussed by Iain Shepherd, Emory University, Atlanta, GA, USA) encodes a subunit of the transcriptional mediator complex (TRAP), which, when mutated, results in a significant reduction of enteric neurons and in defects of other vagal neural crest derivatives. Interestingly, analysis of *lessen* mutants indicates that the gut endoderm plays a crucial role in the early development of the zebrafish ENS, thus providing additional evidence for the interdependence of the different cell types that form the gut during organogenesis (Iain Shepherd). We are confident that by the time of the next meeting (provisionally scheduled for 2008), further analysis of zebrafish ENS mutants will provide new and exciting insights into the developmental mechanisms of this branch of the autonomic nervous system. In parallel with the zebrafish experiments, similar screens were reported in mice. One of them (presented by William Pavan, NIH, Bethesda, MD, USA) was carried out in a sensitised genetic background ($Sox10^{lacZ}$), and involved two mouse strains that facilitate mapping of both dominant and recessive phenotypes. Several novel loci were uncovered that affect various aspects of reporter (*lacZ*) expression. The molecular and phenotypic characterisation of these mutations is in progress, but this report demonstrates the feasibility of this approach for identifying novel loci regulating the development of neural crest derivatives.

Ret and *Sox10* in ENS development

Many of the talks provided new insight into the role of *Ret*, *Sox10* and related genes in the developing ENS. These talks included structure-function analyses suggesting that the RET transmembrane domain plays an important role in receptor dimerisation in the setting of *MEN2A*-activating mutations (*RET* – Human Gene Nomenclature Database) (Carlos Ibáñez, Karolinska Institute, Stockholm, Sweden). Evidence was also presented that the RET ligand GDNF binds the extracellular matrix molecule N-Syndecan and that this interaction is important for mediating the role of GDNF in the tangential migration of cortical interneurons (Mart Saarma, University of Helsinki, Helsinki, Finland). Novel insight into the role of GDNF/GFRα1/RET signalling pathway in ENS development was also provided by new hypomorphic or conditional mutations in RET signalling. This is important because many aspects of RET function have been difficult to evaluate in mice with null *Ret* mutations, as these mutations almost completely eliminate the ENS at early stages of development (Newgreen and Young, 2002). The first example of what the future holds in this area came from the use of the Cre-LoxP system to inactivate *Gfra1* at different stages of embryogenesis (Hideki Enomoto, Riken Center for Development Biology, Kobe, Japan). This analysis demonstrated that *Gfra1* activity is required after E14.5 for ENS precursor migration, proliferation and survival in the distal bowel. These studies also revealed a clear and selective effect of the *Gfra1* deletion on the differentiation of ENS progenitors into neurons. Consistent with this view, analysis of mouse embryos homozygous for a hypomorphic *Ret* mutation (*Ret$^{51/51}$*) indicated an in vivo requirement for RET signalling in neuronal differentiation and axonogenesis (Vassilis Pachnis, National Institute of Medical Research, London, UK). The significance of these presentations goes beyond the understanding of the role of GDNF signalling in ENS development and suggests that the phenotype and complications of HSCR, at least in those cases associated with deficits of RET signalling, result from the combined effect of the distal aganglionosis and the defects in neuronal circuitry in the proximal bowel. Exciting results have also started to emerge from the analysis of mouse strains expressing variants of RET with specific amino acid substitutions that affect distinct intracellular signalling pathways (Masahide Takahashi, Nagoya University, Nagoya, Japan). The importance of understanding the role of intracellular signalling pathways in ENS function was also highlighted by the description of a new mutant mouse strain with abnormal PKA signalling and intestinal dysmotility.

Understanding the role of RET signalling in human HSCR is an ongoing challenge (Emison et al., 2005). Although *RET* is the most commonly identified HSCR-associated gene in humans, mutations in other genes involved in ENS development have also been described (including *ECE1*, *EDN3*, *EDNRB*, *GDNF*, *NRTN*, *SOX10* and *ZFHX1B*). However, HSCR does not follow the conventional norms of Mendelian genetics and is a multifactorial condition with as yet unknown genetic and environmental factors implicated (Chakravarti, 2001). Despite this, one theme emerging over the years and reinforced at this meeting is the central role of *RET* signalling in the development of this condition 'possibly in every HSCR patient' (Aravinda Chakravarti, Johns Hopkins University School of Medicine, Baltimore, MD, USA). This hypothesis is further supported by the realisation that relatively common non-coding variants in the *RET* locus are associated with HSCR susceptibility and make significant contributions to risk. Such variants are either within conserved enhancer-like sequences (Aravinda Chakravarti) or within other non-coding regions (Isabella Ceccherini, Istituto Giannina Gaslini, Genova, Italy),

and can influence the levels of RET protein. In light of this, it was very interesting to hear about animal model systems (mice and zebrafish) that could be used for the evaluation of *RET* promoter mutations (as presented by Elizabeth Grice and Andrew McCallion, Johns Hopkins University School of Medicine, Baltimore, MD, USA). These models promise quick advancements in the study of *RET* expression and highlight the potential implications of promoter mutations in the development of HSCR.

Despite the key role of RET signalling in the ENS, interactions between different signalling pathways (including those of endothelin 3 and Sox10) crucially influence the development of the vertebrate ENS and the pathogenesis of HSCR. Several presentations were devoted to dissecting in more detail interactions between known genes or to identifying novel partners that increase HSCR susceptibility. Thus, genetic interactions between *Sox10* and *Edn3* were shown to influence not only the development of the ENS but also other neural crest derivatives such as melanocytes (Nadege Bondurand, Hôpital Henri Mondor, Creteil, France). The crosstalk between *Sox10* and *Sox8* was also presented using state-of-the-art gene targeting technology in mice (Michael Wegner, Universität Erlangen-Nürnberg, Erlangen, Germany). It has emerged from these studies that *Sox10* and *Sox8* display equivalent functions, but contribute differentially to ENS development in vivo in accordance with their corresponding levels of expression. Finally, participants heard evidence of genetic interactions between *Sox10*, *Gdnf*, *Gfra1* and other genetic loci mapped in congenic strains that show different phenotypic effects of *Sox10* mutations (Michelle Southard-Smith, Vanderbilt University School of Medicine, Nashville, TN, USA). The identification and characterisation of the products of these loci will provide new clues as to how *Sox10* regulates the transition of multi-lineage neural crest progenitors to lineage commitment and differentiation in the peripheral nervous system (Robert Kelsh, University of Bath, Bath, UK).

In parallel with these genetic studies were reports that *Sox10* regulates the expression of several small RhoGTPases and that neural crest-specific deletions of both *Cdc42* and *Rac1* cause a reduction in all neural crest derivatives, including the ENS (Lukas Sommer, Swiss Federal Institute of Technology, Zürich, Switzerland). The role of the small GTPase signalling network in enteric neural crest cell migration was also the subject of a poster, in which pharmacological inhibitors were used to block the activity of specific GTPases or their downstream mediators (Richard Anderson and Heather Young). Interestingly, this approach allowed the uncoupling of the axonal growth and neural crest cell migration, suggesting that the two processes are controlled independently by the gut microenvironment. The crucial role of the Cdc42 GTPase was also elegantly demonstrated by combining in vivo, four-dimensional (3D+time) confocal imaging with targeted molecular perturbation to demonstrate that chick vagal neural crest cells migrate to the gut in a programmed manner. Analysis of the migratory pattern showed that the initial group of emerging vagal neural crest cells form a wide stream that maintains very directed cell trajectories. By contrast, later emerging cells form follow-the-leader chain-like arrays and maintain contact with neighbours through filopodial extensions. Interestingly, when the ability of the neural crest cell to form filopodia and make cell-cell contacts is inhibited by perturbation of Cdc42 function, the chain assemblies are disrupted, individual cell trajectories are less directional and cells are delayed in reaching the branchial arches (Paul Kulesa, Stowers Institute for Medical Research, Kansas City, KS, USA).
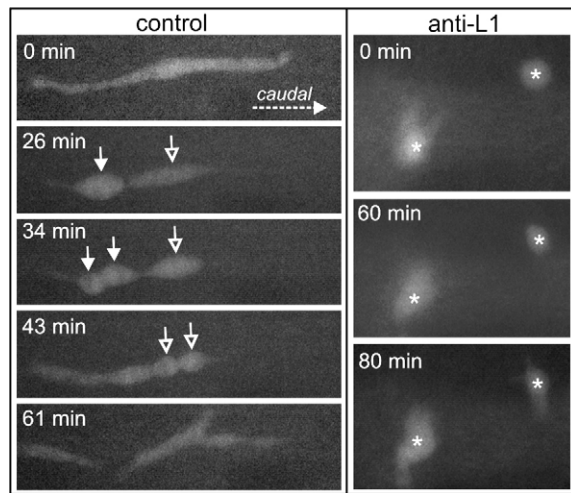
Fig. 2. GFP⁺ cells in explants of hindgut (taken from *Ret*^TGM/+ embryos) maintained in control conditions or exposed to an L1 function blocking antibody for 18 hours. In the control explant, the cells are elongated and migrate in chains, except when undergoing cell division. After dividing, the daughter cells quickly become elongated and resume migration as a chain. When the activity of the cell-adhesion molecule L1 is perturbed, many solitary cells are observed (asterisks) that are round and do not migrate (Anderson et al., 2006). Image kindly provided by Richard Anderson and Heather Young (University of Melbourne, Melbourne, Australia).

Promising new approaches

Stunning views of migrating enteric neural crest have been made possible by the expression of fluorescent proteins in early ENS progenitors (Druckenbrod and Epstein, 2005; Young et al., 2004). So far, most of these studies have focused on imaging embryonic gut from wild-type embryos and understanding the normal patterns of migration of vagal neural crest. However, comparative video microscopy of guts derived from wild-type and mutant embryos promises to provide new and exciting information regarding specific cellular changes associated with mutations affecting the ENS (Miles Epstein, University of Wisconsin, Madison, USA; Richard Anderson and Hideki Enomoto) (Fig. 2). The long-term goal of course is to understand how the building blocks of the ENS come together to form functional reflexes and circuits that control the complex activity of the bowel. Work presented at this meeting on the potential roles of neurexins and neuroligins promises to provide significant advances in this area (Michael Gershon, Columbia University, New York, NY, USA). Establishing improved methods for assessing ENS function in humans, mice and other model organisms is also crucially important, especially in light of the ongoing efforts to restore ENS function by transplantation. Impressive combinations of video imaging and sophisticated mathematical approaches in conjunction with chemical inhibitors now allow the analysis of intestinal motor function in mice as early as E18 (Joel Bornstein, University of Melbourne, Australia). Other studies in organotypic gut cultures demonstrated that intestinal muscle function can be assessed as early as E15 in mice (Kent Sanders, University of Nevada School of Medicine, Reno, NV, USA). As many important mutations are perinatal lethal, these analytical methods should provide novel insight into ENS and intestinal function in mice that could not previously be evaluated.

Although the ongoing basic research into ENS development is fascinating in its own right, one of the long-term goals of this work is to provide new methods to reduce human morbidity and mortality associated with digestive diseases (Young, 2005). For this reason, we were delighted to see a significant effort by several groups of investigators to understand the behaviour of neural crest stem cells and to determine whether transplantation of ENS or other neural progenitors into the aganglionic or abnormally innervated bowel will allow the restoration of ENS function (specifically, the laboratories of Mai Har Sham; Pankaj Jay Pasricha; Nikhil Thapar; Gudrun Gossrau and Oliver Brustle; Jack Mosher and Sean Morrison; Ulrich Rauch and Karl-Herbert Schäfer; and Richard Lindley and Simon Kenney). Although there are many hurdles to overcome for this to be successful, the work provides new hope that we will some day have novel treatment options for people with serious defects in ENS structure and function.

Conclusion

This is an exciting time for researchers interested in ENS development. Whereas the past decade has brought tremendous insight into the molecular and cellular mechanisms needed to form the ENS, the research presented at meeting in New York City provided new hope for the future and set forth challenges for the next 10 years.

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