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Effects of different regions of the developing gut on the migration of enteric neural crest-derived cells: A role for Sema3A, but not Sema3F

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Abstract

The enteric nervous system arises from vagal (caudal hindbrain) and sacral level neural crest-derived cells that migrate into and along the developing gut. Data from previous studies have suggested that (i) there may be gradients along the gut that induce the caudally directed migration of vagal enteric neural precursors (ENPs), (ii) exposure to the caecum might alter the migratory ability of vagal ENPs and (iii) Sema3A might regulate the entry into the hindgut of ENPs derived from sacral neural crest. Using co-cultures we show that there is no detectable gradient of chemoattractive molecules along the pre-caecal gut that specifically promotes the caudally directed migration of vagal ENPs, although vagal ENPs migrate faster caudally than rostrally along explants of hindgut. Exposure to the caecum did not alter the rate at which ENPs colonized explants of hindgut, but it did alter the ability of ENPs to colonize the midgut. The co-cultures also revealed that there is localized expression of a repulsive cue in the distal hindgut, which might delay the entry of sacral ENPs. We show that Sema3A is expressed by the hindgut mesenchyme and its receptor, neuropilin-1, is expressed by migrating ENPs. Furthermore, there is premature entry of sacral ENPs and extrinsic axons into the distal hindgut of fetal mice lacking Sema3A. These data show that Sema3A expressed by the distal hindgut regulates the entry of sacral ENPs and extrinsic axons into the hindgut. ENPs did not express neuropilin-2 and there was no detectable change in the timetable by which ENPs colonize the gut in mice lacking neuropilin-2.

Keywords: Neural crest; Migration; Gastrointestinal tract; Semaphorin 3A; Neuropilin-1; Semaphorin 3F; Neuropilin-2

Introduction

The enteric nervous system (ENS) is an extensive system of neurons and glial cells within the gut wall. In most gut regions, the ENS is capable of independently modulating or controlling many gut functions including motility and secretion, although the CNS can also influence these reflexes (Gershon, 2005; Furness, 2006). The vast majority of enteric neurons and glial cells arise from neural crest cells in the caudal hindbrain termed "vagal" neural crest cells, although sacral neural crest cells also contribute some neurons and glia, principally in the distal hindgut (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Kapur et al., 1992; Durbec et al., 1996; Burns and Le Douarin, 1998; Kapur, 2000; Burns, 2005; Anderson et al., 2006;). After emigrating from the hindbrain, vagal enteric neural precursors (ENPs) migrate into the foregut, and then along the gut, within the mesenchyme, to colonize the entire gastrointestinal tract (Baetge and Gershon, 1989; Kapur et al., 1992; Newgreen et al., 1996; Burns and Le Douarin, 1998; Young et al., 1998; Natarajan et al., 1999; Conner et al., 2003). In mice it takes over 4 days, or approximately 25% of the gestation period, for ENPs to colonize the entire gastrointestinal tract (Kapur, 1999; Young and Newgreen, 2001), and in humans the process takes approximately 3 weeks (Fu et al., 2003; Wallace and Burns, 2005). As the gut is elongating during the colonization process, ENPs are thought to migrate further than

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any other population of neural crest-derived cells (Newgreen et al., 1996; Newgreen and Young, 2002). During the developmental stages that ENP migration occurs, gut morphogenesis has commenced, and some parts of the gut have begun to adopt distinct regional identities.

Migration of ENPs through the gut is regulated by multiple factors, including the number of ENPs (Burns et al., 2000; Simpson et al., 2007), growth of the gut (Landman et al., 2003) and molecules expressed by the gut mesenchyme including glial cell line-derived neurotrophic factor (GDNF), endothelin-3 and netrins (Jiang et al., 2003). GDNF is expressed by the gut mesenchyme, and studies in vitro have shown that GDNF is chemoattractive to ENPs (Young et al., 2001; Natarajan et al., 2002; Iwashita et al., 2003). At the developmental stages at which ENPs are colonizing the small intestine and caecum of fetal mice, the expression of GDNF is maximal in the pre-caecal midgut and caecum (Natarajan et al., 2002). This has led to the suggestion that GDNF might promote the colonization of the small intestine by ENPs (Natarajan et al., 2002). The expression of GDNF in the hindgut is lower than that of the caecum (Natarajan et al., 2002), but it is unknown how this impacts on the migration of ENPs.

Endothelin-3 is expressed in the developing gut, and its receptor, endothelin receptor B (EDNRB), is expressed by migrating ENPs (Leibl et al., 1999; Lee et al., 2003). The expression of endothelin-3 in the developing gut is highest in the caecum than other gut regions (Leibl et al., 1999; Barlow et al., 2003). As endothelin-3 reduces the chemoattractive effects of GDNF in vitro, it has been proposed that exposure to the caecum might alter the migratory properties of ENPs (Barlow et al., 2003; Kruger et al., 2003). Furthermore, endothelin-3 also promotes the proliferation and inhibits the differentiation of ENPs, but does not appear to affect ENP survival (Hearn et al., 1998; Wu et al., 1999; Bondurand et al., 2006). As signaling via EDNRB receptors is only required for normal ENS development between E11.5 and E12.5, which is when ENPs are colonizing the caecum (Shin et al., 1999; Woodward et al., 2000), it has also been proposed that migration through the caecum is important for colonization of the hindgut by ENPs. However, it is currently unclear whether ENPs that have not been exposed to the caecum can colonize the hindgut as well as ENPs that have been exposed to the caecum.

ENPs derived from sacral neural crest cells migrate to the vicinity of the distal hindgut and then undergo a waiting period for several days and do not enter the distal hindgut until shortly before or shortly after vagal ENPs colonize this region (Burns and Le Douarin, 1998; Kapur, 2000; Druckenbrod and Epstein, 2005; Anderson et al., 2006; Nagy et al., 2006). Sacral ENPs are thought to migrate into the hindgut of chick embryos along the axons of extrinsic neurons (Burns and Le Douarin, 1998; Burns, 2005). In a number of regions of the developing nervous system where migrating cells or axons halt for a period of time before navigating to their correct target, it has been demonstrated that the waiting periods are mediated by the expression of repulsive cues (Watanabe et al., 2006; Renzi et al., 2000). In chick embryos, Sema3A is expressed by the mesenchyme of the distal large intestine, and neuropilin-1 is expressed by axons of neurons in the nerve of Remak, which is located parallel to, but outside, the gut in birds (Shepherd and Raper, 1999). The axons of neurons in Remak's nerve undergo a waiting period and do not penetrate the outer layers of the gut wall until the expression of Sema3A recedes to more internal layers, and *in vitro*, Sema3A is repulsive to the neurites of neurons in the nerve of Remak (Shepherd and Raper, 1999). It has therefore been proposed that Sema3A regulates the entry of intrinsic axons into the hindgut (Shepherd and Raper, 1999), although to date there are no *in vivo* data. As sacral ENPs accompany extrinsic axons into the hindgut (Burns and Le Douarin, 1998), it has also been suggested that Sema3A directly or indirectly, via its repulsive effect on extrinsic axons, regulates the entry of sacral ENPs into the hindgut (Shepherd and Raper, 1999; Young and Newgreen, 2001; Newgreen and Young, 2002; Burns, 2005; Burns and Thapar, 2006). Sema3A-neuropilin-1 signaling has not yet been shown to influence the migration of ENPs, although Sema3A has been shown to be repulsive to trunk and cranial neural crest cell populations in vitro (Eickholt et al., 1999) and to be essential for the correct migration of sympathetic neuron precursors (Kawasaki et al., 2002). Recent studies in vivo have shown that neuropilin-2 and its ligand, Sema3F, influence the migration pathways of some cranial and trunk neural crest cells (Osborne et al., 2005; Yu and Moens, 2005; Gammill et al., 2006a,b). Although Sema3F is expressed at high levels in the distal hindgut of rat embryos (Giger et al., 1998), the involvement of Sema3F-neuropilin-2 signaling in ENP migration has not previously been investigated.

In this study we tested a number of theories about the guidance of ENPs along the developing gut that have been proposed based on data from previous studies. The rate of migration of ENPs along explants of pre-caecal and post-caecal gut was compared to determine whether the migratory speed of ENPs differs in different regions of the gastrointestinal tract, and the abilities of ENPs derived from the pre-caecal gut or the caecum to migrate along explants of hindgut was also compared. To determine if there are gradients along the pre- and post-caecal gut that influence the migration of ENPs, we used co-cultures to compare the effect of direction of migration on the rate of migration of ENPs. Finally, we examined the expression of Sema3A and Sema3F and their receptors, neuropilin-1 and neuropilin-2, in the gut of fetal mice, and the entry of sacral ENPs into the distal hindgut in mice lacking members of the Sema3A or Sema3F signaling pathways.

Materials and methods

Animals

All mice were on a C57/BL6 background. Timed pregnant mice were killed by cervical dislocation and the embryos removed. The morning on which a copulatory plug was observed was designated E0.5. All procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Ret-TGM mice: *Ret*-TGM mice have had cDNA encoding tau-EGFP-myc (TGM) inserted into the first coding exon of the *Ret* gene (Enomoto et al., 2001) and all enteric neural crest-derived cells in the gut of $Ret^{TGM/+}$ embryos express GFP (Young et al., 2004). The genotype of adult Ret^{TGM} mice was determined by PCR using the primers and conditions reported previously (Enomoto et al.,