Choices Choices: Regulation of Precursor Differentiation during Enteric Nervous System Development

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Abstract

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system and forms a complex circuit of neurons and glia that controls the function of the gastrointestinal (GI) tract. Within this circuit there are multiple subtypes of neurons and glia. Appropriate differentiation of these various cell subtypes is vital for normal ENS and GI function. Studies of the pediatric disorder Hirschprung's Disease (HSCR) have provided a number of important insights into the mechanisms and molecules involved in ENS development, however there are numerous other GI disorders that potentially may result from defects in development/ differentiation of only a subset of ENS neurons or glia. Our understanding of the mechanisms and molecules involved in this process is far from complete. Critically, it is unclear at what point the fates of enteric neural crest cells (ENCCs) become committed to a specific subtype cell fate and how these cell fate choices are made. We will review our current understanding of ENS differentiation and highlight key questions that need to be addressed in order to gain a more complete understanding of this biological process.

Keywords

Neural Crest; ENS; Differentiation; Transcriptional Regulation

Introduction

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system. The ENS is responsible for regulating peristalsis, blood flow, and water and electrolyte transport in the gut (1, 2). ENS development is a coordinated process in which neural crest derived ENS precursors must migrate from specific axial locations to and then along the gut. Subsequently they differentiate into the various types of neurons and glia that make up the ENS. Defects in ENS precursor migration, proliferation and differentiation have been shown to lead to hypo and aganglionosis phenotypes in several model systems. In human's intestinal aganglionosis, when present in newborns, leads to the gastrointestinal (GI) disorder Hirschprung's Disease (HSCR) (3–7)

Differentiation of the enteric neural crest derived cells (ENCCs) is one of the key processes in the formation of a fully functional ENS. While studies have begun to elucidate the

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complexities behind this process there are still significant gaps in our understanding. The ENS is a complex system that is made up of up to 17 different subtypes of neurons (1). How this diversity in neuronal subtypes is generated is one the central unanswered questions in the field. Several studies have shown that ENS neurons and glia can be traced back to specific axial populations of neural crest cells but it is unclear exactly when the fate of these neural crest cells becomes determined to generate ENCCs (4, 8–11). It is also unclear at what stage during ENS development these ENCCs become fated to generate a specific subtype of enteric neuron or glia. Potentially, this could be a stochastic process only occurring when ENCCs migrate along the gut and is completely dependent on the ENCCs' final location within the GI tract. At the other extreme, individual ENCCs could be fated to become specific subtypes during the formation of the ENCC population in the pre-migratory/pre-enteric neural crest (Figure 1).

Understanding the processes and mechanisms that regulate differentiation of the various ENS subtypes will not only help us to understand the development of the ENS but may also help us gain a better understanding of the pathologies of various GI disorders. While human aganglionosis disorders have been extensively studied, pathological analysis of patients has been limited to looking for the presence or absence of neurons in the gut. Because there are a large number of GI motility disorders that present in the clinic with no obvious underlying cause, it is quite plausible that some of these conditions result from the absence/loss of a specific subset of ENS neurons or glia (12). A better understanding of the mechanisms involved in the development of subtypes of enteric neurons and glia may give significant insights into the etiologies of some currently unexplained GI motility disorders.

Early ENS Specification of the Neural Crest

One major unanswered question in ENS development is how early during embryogenesis does ENS specification occur? The key event appears to be the formation of specific axial populations of neural crest cells (NCCs). Classic chick-quail chimera studies indicate that cells within the vagal neural crest are sufficient to form most of the ENS (13, 14). In the zebrafish model system, the ENS is completely formed from vagal neural crest cells, however in mammals and chicks the ENS is formed not only from the vagal cells but is also derived in part from sacral neural crest cells (15–18). These vagal and sacral neural crest cells appear to develop in a semi-cell autonomous manner indicating there is some level of specification that occurs early on during neural crest formation. When vagal and sacral neural crest cells were reciprocally transplanted to the other axial location, transplanted cells went on to form structures appropriate for their new axial location. However sacral crest cells were not as efficient at generating ENS neurons and glia as vagal crest cells (8, 9). Similarly, when vagal NCCs were transplanted to the sacral region the transplanted cells followed the normal migration route of sacral cells to the gut but did so earlier and in a much greater number (10). It appears that while there is some flexibility in the axial origin of the neural crest that gives rise to the ENCCs, vagal NCCs are the preferential axial source of NCCs for the ENS and a critical number of NCCs are necessary for normal formation of the ENS (19). When the number of vagal NCC derived ENCC precursors is reduced the rate of ENCC migration along the gut proceeds at a much slower rate (20). This may be due to a lack of cell-cell contact between the low numbers of ENCCs (20). Mathematical modeling

of the process of ENCC colonization of the gut suggests that proliferation differences between the different axial neural crest populations determine their ability to generate a complete ENS (21). These models show that cranial neural crest have a spatially determined proliferative advantage in forming an ENS in comparison to trunk neural crest (21). Given these results, it is clear that distinct populations of NCCs are specified to be ENCCs within the vagal neural crest and these cells are needed to correctly populate the gut with neurons and glia.

It is clear that the spatial organization of the neural crest is important for determining the eventual fate of the ENCCs. One key anatomical structure that affects the early specification of specific vagal neural crest derivatives is the dorsal aorta. Expression of BMP4 and 7 from the dorsal aorta induces the expression of pro-sympathetic neural genes. Inhibition of BMP expression from the dorsal aorta prevents sympathetic neuron formation (22, 23). The expression of BMP by the dorsal aorta gives rise to a concentration gradient of BMP ligand in this region and this means that as neural crest cells migrate towards the gut they are exposed to varying BMP concentrations. As a result, NCCs may acquire different cell fates within the ventrally migrating stream depending on the length of time and the concentration of BMP ligand to which they are exposed to during their migration. Similarly, there is a gradient of Wnt expression extending from the neural tube laterally. NCCs that express β-catenin, a down stream signaling component of the canonical signaling pathway, at high levels have been shown to form sensory neurons (24, 25). While these concentration gradients have been well studied for sympathetic neurons their specific affects on ENCCs are less clear and needs further study.

The previously discussed embryological chick-quail chimera studies suggest that there is some level of fate determination that is occurring early on in the formation of the neural crest but the molecular basis behind this fate determination is unclear. At the earliest stage of neural crest specification, the transcription factors FoxD3 and Sox10 are expressed by the neural crest at the stage when it arises from the neuroepithelium (26, 27). Functionally, FoxD3 has been shown to be important in the early selection of neuronal cell fates as opposed to non-neuronal cell fates within the neural crest (27). Sox10 is also expressed throughout neural crest development in the vagal and sacral regions and continues to be expressed in ENCCs when they reach the gut and begin migrating along it (11, 16). Sox10 along with Pax3 induce expression of the tyrosine kinase RET, a key gene in ENS development and an early ENCC marker that has been shown to be the primary gene associated with HSCR (16, 28–30). RET's initial function is to promote the survival of ENCCs, acting as the signal transducing component of the GDNF receptor along with its co-receptor GFR(alpha)1 (31, 32). Phox2b is another early marker whose expression is dependent on Sox10 (33, 34). Not only is Phox2b Sox10-dependent, but it also is expressed by ENCCs throughout their migration along the gut (34). Phox2b also appears to be important for the expression of RET as well as for the expression of the basic helix-loop-helix (bHLH) transcription factors Ascl1 and Hand2 (33, 35). Furthermore, in addition to the previously mentioned transcription factors, Hox genes are involved in determining the fate of neural crest cells. Studies have shown that the different axial populations of NCCs express different Hox genes, dependent on their axial origin, and this NCC Hox gene

expression affects their cell fate (36, 37). Vagal neural crest cells express HoxB3 and this expression may play a role in determining the ENCC fate for a subset of these cells (38).

Several of the identified transcription factors are expressed in both the pre-migratory and migratory vagal neural crest precursors, his suggests that these factors potentially have an important role in ENCC/ENS fate determination. However, the full complement of transcription factors that are responsible for the selection of ENS precursor cell fate as opposed to other neural crest cell derivatives has not been elucidated. In addition, while these transcription factors are required for ENS development, it is not clear whether any of these genes confers a specific ENCC cell fate for an individual NCC or whether their function is simply to confer a commitment to a neural/glia cell fate.

Post Neural Crest Differentiation Control

Once the ENCCs begin to enter the gut their environment changes significantly and the signals they receive become even more important for maintaining their proliferative potential and for determining their eventual ENS cell fate. In vitro studies have shown that sympathetic neuroblasts take on enteric characteristics when cultured with gut monolayers while enteric neuroblasts take on sympathetic characteristics when cultured with dorsal aorta monolayers (39). This clearly demonstrates the importance of environmental signals in regulating ENS differentiation (39). As the ENCCs enter the gut, they continue to express Sox10, RET, and Phox2b (11, 16, 31–33). Sox10 is critically important for maintaining the progenitor state of ENCCs and it appears that in mice Sox10 expressing cells maintain neurogenic potential into adulthood (40, 41). Sox10 also influences the expression of several other proteins shown to be involved in ENCC development including Ascl1, a bHLH transcription factor that actually represses Sox10 expression and promotes expression of pro-neural genes (26). This Sox10 driven expression of Ascl1 appears to be modulated by the notch pathway as a component of the notch signaling pathway, Hes1, represses Ascl1 expression (39). As a result, notch signaling is potentially important in maintaining the ENCCs progenitor potential at least for a subset of ENCCs though this requires further investigation (42, 43).

Sox10 also influences the expression of the G-Protein coupled receptor EDNRB, which has been shown to be important for complete ENCC colonization of the gut (44–46). EDNRB and its ligand endothelin-3 (ET-3), seem to prevent neuronal differentiation in ENCCs and helps maintain their potential to colonize the rest of the gut (45, 47, 48). Another gene that appears to prevent neuronal differentiation in favor of proliferation is sonic hedgehog (shh). shh is expressed in the developing gut endoderm. shh modulates ENCC responsiveness to GDNF promoting cell proliferation and migration while attenuating/inhibiting neuronal differentiation (49, 50).

Many of the genes that have been shown to be critical to ENS development appear to be expressed throughout this developmental process. Interestingly, many of these signaling pathways involved early in neural crest development often have additional/alternate functions later in ENCC specification. One example is the BMP family of proteins, specifically BMP4. BMP4 is involved in differentiation of neural crest-derived cells into neurons in vitro (51–53). It appears that ENCCs have a dose dependent response to BMP

signaling, as low concentrations of BMP promote an ENCC to stay in an undifferentiated proliferative state while high concentrations promote neurogenesis (52, 53). One way in which BMP may influence neurogenesis is through its interaction with Ascl1 (54, 55). BMP2 decreases the stability of Ascl1 leading to an inhibition of certain pro-neural genes (56–58). Ascl1 appears to promote the expression of the transcription factor Phox2a and together these two transcription factors promote the expression of a subset of pro-neural genes. Previous studies looking at autonomic nervous system development have shown Ascl1 couples the expression of general neuronal markers with subtype specific markers (59, 60). Ascl1 is also critically important for the development of esophageal neurons as Ascl1$^{-/-}$ mice have perturbed gangliogenesis in the esophagus (61). This suggests that other basic helix-loop-helix (bHLH) pro-neural transcription factors are also involved in ENS development (62). One potential candidate bHLH transcription factor, expressed in ENCCs, is Hand2, whose expression is also regulated by BMP in vitro (63). Furthermore Hand2 has been shown to regulate expression and function of phox2a and phox2b genes in the development of sympathetic nervous system (SNS) and couples neurogenesis and cell type gene expression in the SNS (64–66).

Another gene that appears to have a bimodal role in ENS development is FoxD3. While the ENS glial population comes from the same ENCC precursor pool as the ENS neurons, in mice glial development lags behind the development of the neurons as glial markers are not seen in the early migrating ENCC chains as opposed to neuronal markers, which are observed (67–69). This is not the case however in the chick where ENS neuronal and glial differentiation occurs concurrently in the migrating chains of ENCC (70). FoxD3 has been shown to promote gliogenesis in the ENS, as well as influence proliferation and neural patterning (71). Notch signaling and the bHLH transcription factor Hand2 also affect the ENS glial cell development (72). However Hand2 appears to indirectly affect glial development, because the glial phenotype seen in Hand2–/– mice results from an overall reduced size of the initial ENCC progenitor pool rather than any specific effect on gliogenesis cell fate determination (73). Sox10 also is expressed in glia cells into adulthood allowing these cells to maintain their neurogenic potential (41, 74) These results clearly indicate that there are several regulatory pathways that function to maintain the cell fate potential in the ENCCs. However, it is less clear when these cells become further committed to a specific enteric neuron subtype or glial cell fate and how the switch from proliferating ENCC to committed neural or glial precursor is regulated.

Subtype Specification

Depending on the species, differentiation of ENCCs begins at different times during the migration process. In zebrafish, there appears to be two main waves of neuronal differentiation that occur at 72 and 96 hours post fertilization (hpf), respectively(75). This brings up an interesting problem as at 72hpf the ENCCs have migrated along the length of the gut, but a subset continue to proliferate and another group needs to circumferentially migrate around the gut to completely populate it. This means that only a specific subset of cells differentiate in this initial wave. While differentiation does not begin until after the anterior posterior migration along the gut is completed in zebrafish, mouse ENCCs in the anterior portion of the gut begin to differentiate before ENCCs have migrated to the

posterior end of the gut (69). In both fish and mice, differentiation of all neuronal and glial subtypes does not occur at the same time. This indicates that ENCCs must be temporally restricted in the cell types to which they can give rise.

While there are specific waves of differentiation seen in the ENCCs of model organisms pan-neuronal markers appear much earlier in ENS development. These markers begin to appear as early as E10.5 in mice and in zebrafish between 24 and 48 hpf (76–78). These early cells are electrically active as early as E11.5 in mice, indicating that these cells have begun exhibiting both the molecular and physiological characteristics of neurons (79).

While certain pan-neuronal markers begin to show up early in ENS development the presence of specific markers for nitronergic (nNOS), serotonergic (5-HT), cholinergic (ChAT, VAChT), dopaminergic (DBH, TH) neurons appear at varying times during ENS formation (75, 80–85). Similarly various other neuronal and pan-neuronal markers (IkCa, CGRP, calbindin, calretinin, VIP, substance P) appear at varying times in ENS development (Figure 2) (80–82, 84, 85). The earliest expressed neuronal cell type specific marker in mice and zebrafish is nNOS, appearing around E11.5 in mice and between 48 and 72hpf in zebrafish (75, 80). Calbindin and IkCa channels also appear at E11.5 in mice but many other differentiation markers are absent (80). Substance P, VIP, and 5-HT neurons appear around E14 in mice while CGRP is not present until E17 (81, 82). Molecules involved in the synthesis of acetylcholine are present in mice between E10–12 but the ChAT and VAChT markers themselves do not appear until around E18.5 (83). Similarly in zebrafish expression of the markers for VIP, calbindin, CGRP, 5-HT and others do not appear until later in development between 72 and 96hpf (75, 84).

While it is clear that the markers of these different subtypes begin to appear at different times, it is less clearly understood how a particular subtype is specified in the ENS. So far only a few genes and signaling pathways have been shown to have a specific role in the specification of a specific ENS neuronal subtype. One of these genes, Ascl1, appears to regulate 5-HT ENS neuronal differentiation, as this type of neuron is particularly affected in Ascl1−/− mice (62). However Ascl1 does not appear to be solely responsible for 5-HT ENS neuron development as not all Ascl1 expressing ENCC/ENS neurons are 5-HT positive (86). 5-HT neurons, along with calretenin expressing neurons, are also influenced by norepinephrine transporter (NET) as NET −/− mice have reduced numbers of these neuronal subtypes (87). Neutrophin-3 (NT-3) appears to affect submucosal intrinsic primary afferent neurons expressing CGRP because there are fewer of these neurons in TrkC −/− mice, which is the receptor for NT-3 (88). Hand2 −/− mice also have a complete loss of nNOS and VIP enteric neurons and a significant reduction in the number of calretnin and ChAT enteric neurons (73, 89). It also appears that nNOS formation is influenced by neural activity as ENCCs exposed to tetanus toxin to block neural activity by SNARE mediated vesicle fusion caused a decrease in the number of nNOS enteric neurons(80). Interestingly GDNF-RET signaling can also influence subtype specification as increasing GDNF expression later in ENS development can alter the numbers of certain neuronal subtypes including nNOS neurons (90–92). This may be a result of the influence GDNF has determining when ENCC precursors leave the cell cycle. Different ENS neuronal subtypes arise at varying time points so by altering the stage at which ENCC leave the cell cycle will influence the respective

proportions of different ENS neuronal subtypes (91). Clearly some ENS neuronal subtypes develop later than others so it is possible that the earlier born neurons influence the development of later developing neurons. Furthermore 5-HT expression in the ENS seems to influence the development of dopaminergic, GABAergic, CGRP expressing, and late born nitregic enteric neurons but it is not clear whether 5-HT directly influences their differentiation or it is simply important for their development/survival (93). Together these results indicate that it is a combination of extrinsic and intrinsic signals that lead to ENCC/ENS neuron and glial cell fate determination and differentiation.

Further insights into ENS cell subtype specification can be gained from examining specification in other parts of the peripheral nervous system. Sympathetic neuron expression of DBH is influenced by a combination of factors including Phox2a and Hand2 (94–96). Similarly, Phox2a and Hand2 influence sympathetic neuron expression of TH, however TH neurons respond differently to protein kinase A (PKA) activity as compared to DBH neurons. In TH neurons Phox2a and PKA act independently but in DBH neurons Phox2a and PKA synergistically (94, 97). Finally the cholinergic markers ChAT and VAChT in the parasympathetic system appear to be influenced by the expression of PKA as well as the transcription factor REST (80, 98).

It is clear from looking at studies into subtype specification in other neuronal systems that the formation of these subtypes is a complicated process involving the interaction of multiple transcription factors and signaling pathways. To fully understand the specific molecular combination of signals and factors that influence terminal cell fate specification in the ENS will require the generation of more conditional knockout and over-expression animal models. In addition, a better understanding of the precise lineage relationship between the different ENS neuronal and glial subtypes is still needed if we are to get a more complete understanding of this process. Given that different subtype markers appear at different times it is also possible that the different ENS neuron and glia subtypes may become specified at different times and that ENS development is some combination of the two models presented in Figure 1. Recent technological advances such as the development of the brainbow lineage reporter system will potentially allow us to gain a much clearer understanding of the lineage relationship within the developing ENS (99, 100).

Perspectives

The ENS is a complex, dynamic circuit of neurons and glia that are necessary for normal healthy digestion. Any errors in ENS formation can have drastic consequences for the development of an individual, as evidenced by the aganglionosis phenotype seen in Hirschprung's disease. Furthermore we have barely begun to understand the neuronal basis of many GI disorders. The complex circuit that is the ENS is made up of multiple different subtypes of neurons, each of which are necessary for normal ENS function. Should the proper equilibrium of neuronal subtypes not form there is a risk the whole circuit will not work properly. While we can characterize the aganglionosis phenotype in HSCR comparatively easily, detecting GI disorders that affect only a specific subpopulation of ENS neurons and glia is far more challenging. Clinically the loss/reduction in number of a

specific ENS neuronal subtype potentially could be the cause of many GI disorders that as of present have no known cellular basis.

While our understanding of the differentiation and development of the ENS has grown greatly over the past few years, there is still much that still needs to be determined. As we have pointed out in this review, while the appearance of subtypes has been well characterized the actual molecular basis for how this specification process occurs both cellularly and mechanistically is not well understood. How early does enteric neuron and glia subtype specification begin? When are ENCCs committed to a specific neuronal subtype fate? Is it extrinsic or intrinsic factors that determine a specific subtype specification? All of these questions and more will need to be answered in order to begin to gain a more complete understanding of the complex process of ENS formation.

Acknowledgments

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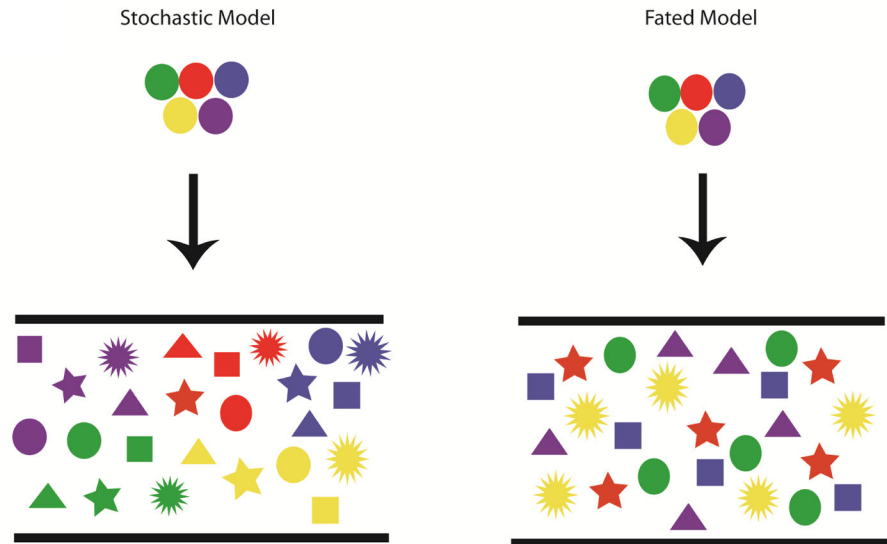
Figure 1.
Models of ENCC Differentiation. There are two models that could explain the differentiation of the ENCCs into the various subtypes in the gut. The first is a stochastic model in which the fate of the ENCCs is not specified until they reach the gut. In this model any kind of ENS cell subtype can come from any ENCC depending on where it ends up in the gut. In the fated model ENCCs are fated to become specific subtypes early in development and cells derived from one specific ENCC are all fated to become a specific subtype. The different colored shapes indicate different subtypes of enteric neurons and glia.
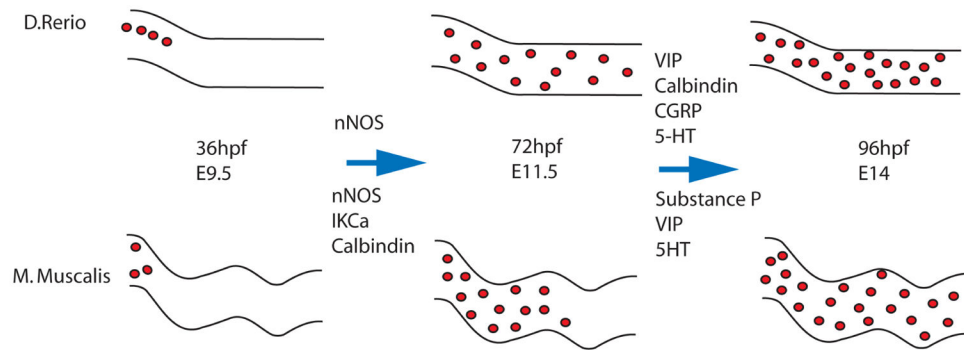
Figure 2.
Schematic diagram of ENCCs populating the guts of D. Rerio and M. Muscalis. ENCCs enter the gut at 36hpf and E9.5 in mouse and zebrafish respectively. nNOS appears in zebrafish between 48–72hpf and around E11.5 in mice. IKCa and calbindin also appear in mice around E11.5. at 72hpf ENCCs have populated the posterior of the gut in zebrafish and a first wave of differentiation has occurred while ENCCs are still migrating to the posterior in mice. A 96hpf the second differentiation wave has occurred in zebrafish while at E14 ENCCs have populated the posterior gut in mice. Between 72 and 96hpf VIP, calbindin, CGRP, and 5HT appear in zebrafish. Substance P, VIP, and 5HT appear at E14 in mice while CGRP doesn't appear until much later at E17. ChaT and VAChT also do not appear until later in mouse development at E18.5.