Published in final edited form as:
Neurochem Int. 2012 November ; 61(6): 839–847. doi:10.1016/j.neuint.2012.01.028.

CELL DEATH AND THE DEVELOPING ENTERIC NERVOUS SYSTEM

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Abstract

This review discusses current knowledge about cell death in the developing enteric nervous system (ENS). It also includes findings about the molecular mechanisms by which such death is mediated. Additional consideration is given to trophic factors that contribute to survival of the precursors and neurons and glia of the ENS, as well to genes that, when mutated or deleted, trigger their death. Although further confirmation is needed, present observations support the view that enteric neural crest-derived precursor cells *en route* to the gut undergo substantial levels of apoptotic death, but that once these cells colonize the gut, there is relatively little death of precursor cells or of neurons and glia during the fetal period. There are also indications that normal neuron loss occurs in the ENS, but at times beyond the perinatal stage. Taken together, these findings suggest that ENS development is similar is some ways, but different in others from extra-enteric areas of the vertebrate central and peripheral nervous systems, in which large-scale apoptotic death of precursor neurons and glia occurs during the fetal and perinatal periods. Potential reasons for these differences are discussed such as a fetal enteric microenvironment that is especially rich in trophic support. In addition to the cell death that occurs during normal ENS development, this review discusses mechanisms of experimentally-induced ENS cell death, such as those that are associated with defective glial cell-line derived neurotrophic factor/Ret signaling, which are an animal model of human congenital megacolon (aganglionosis; Hirschsprung's disease). Such considerations underscore the importance of understanding cell death in the developing ENS, not just from a curiosity-driven point of view, but also because the pathophysiology behind many disorders of human gastrointestinal function may originate in abnormalities of the mechanisms that govern cell survival and death during ENS development.

Keywords

neural crest precursors; migration; post-mitotic; neurons; glia; GDNF/Ret/GFRα1; BMP/Smad; NT-3/TrkC; GGF-2/ErbB3; apoptosis; TUNEL; Bax; BCl-X_L; PI 3 kinase; Akt; autophagy; 5-HT4; HIPK2; aganglionosis; HSCR; gut

Introduction

This review integrates current knowledge about the occurrence and mechanisms of cell death during development of the enteric nervous system (ENS). Interest in this subject has

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been spurred by recent advances that point to a role for cell death in Hirschsprung's disease (HSCR), a major developmental disorder of human infants characterized by aganglionosis of the colon. The studies reviewed here reveal that cell death during ENS development is similar in some ways, but strikingly different in others from the cell death that accompanies development of other regions of the nervous system. We first provide a brief overview of cell death in the developing nervous system as well as a brief summary of the key steps in ENS development. We then describe what is known about normal and pathologic death of cells at various stages of development of the ENS. Finally, we discuss various issues raised by these findings as well as what studies they suggest for the future.

Developmental neuron death: An overview

Developmental death of post-mitotic neurons

It has been long established that post-mitotic neurons in vertebrates undergo cell death during normal development (Hamburger, 1992). This occurs in many populations within the peripheral and central nervous systems and affects on average about 50% of the neurons that are generated (Oppenheim, 1991). In most cases examined, such death appears to be apoptotic in nature (Roth and D'Sa, 2001). Caspase inhibitors, deletion of genes required for apoptotic death, or over expression of genes that suppress apoptosis thus usually prevent cell death during nervous system development. Alternative developmental death mechanisms, however, such as autophagy or necroptosis have been described but, at present, the roles that these mechanisms play in developing nervous systems are largely unknown (Yuan and Kroemer, 2010).

Survival of newborn neurons is supported by a variety of trophic factors, withdrawal of which causes rapid neuron death (Davies, 2003). Studies of the regulation of cell death in newborn neuronal populations have led to formulation of what is often referred to as the neurotrophic theory (Davies, 1996). According to this model, neuronal targets secrete limiting amounts of trophic factors that support the survival of the neurons that innervate them. The innervating neurons compete for this limited supply and consequently cell death eliminates those that fail to receive enough support. Confirmation for the neurotrophic theory includes observations that neuronal targets produce required trophic factors, that excessive supplies of trophic factors prevent normal developmental neuronal death and that deafferentation of newborn neurons leads to their demise. Much of such evidence has come from studies with sympathetic and sensory neurons that are developmentally derived from the neural crest, although supportive findings also come from studies with populations of CNS neurons. It is important to note in addition, that normal developmental death can occur in populations of newborn neurons that have not yet migrated to their final positions and that are not in contact with their targets (Baydyuk et al., 2011). Such findings support the idea that neurons require support by trophic factors soon after their generation and receive trophic support from local sources before they interact with their targets.

Developmental cell death of astroglia

Although much less studied than in neurons, large-scale developmental cell death has also been documented in populations of CNS astrocytes and astrocyte precursors (Chan-Ling et al., 2009; Krueger et al., 1995). The mechanisms by which astrocyte death is controlled have not been studied in detail.

Developmental cell death of neuroprogenitor cells

In addition to widespread death of post-mitotic neurons and astroglia, a number of studies have also documented large-scale death of proliferating neural stem cells and

neuroprogenitor cells during nervous system development (Boya and de la Rosa, 2005). Death of these cells also appears to be apoptotic in nature (Lindsten et al., 2005).

Potential functions of developmental death in the vertebrate nervous system

The large-scale cell death that occurs in the developing nervous system raises the question as to what purposes it serves. One likely possibility is that in order to assure the proper numbers of neurons needed to form connections and circuits in the maturing nervous system, excess cells are generated and that those that are not required are eliminated (Buss et al., 2006). In any case, it is clear that there are major negative consequences if there is interference with normally occurring death in the nervous system. This can be seen for instance from studies in which deletion of genes required for apoptosis leads to lethality associated with greatly increased numbers of neuroprogenitor cells and post-mitotic neurons (Cecconi et al., 1998; Kuida et al., 1998; Lindsten et al., 2005).

Development of the ENS: An overview

The ENS is derived mostly from streams of proliferating neural crest cells originating from 3 levels of the neuraxis that have been defined mainly in chick embryos and fetal mice. In the chick, the major vagal stream originates from somite levels 1–7 and colonizes the entire length of the gut in a rostro-caudal direction. A second stream originates from the rostral truncal level and colonizes the esophagus and adjacent proximal stomach, while a later stream originating from sacral levels (caudal to somite 28) colonizes the distal gut in a caudal-rostral fashion (Burns, 2005; Burns and Le Douarin, 1998; Hearn and Newgreen, 2000; Kapur, 2000; Le Douarin and Teillet, 1973; Pomeranz and Gershon, 1990; Pomeranz et al., 1991; Serbedzija et al., 1991; Yntema and Hammond, 1954). Colonization of the bowel by enteric neural crest-derived cells (ENCDC) starts in the foregut in mice around E9 −9.5 and terminates in the hindgut by about E14–15 (Gershon 1999; Rothman and Gershon, 1982). As it migrates within the bowel (Kapur 2000; Young and Newgreen, 2001), the ENCDC population (although not every cell) continues to proliferate before differentiating into neurons and glia that coalesce into ganglia that form the myenteric and submucosal plexuses. During this process, neurogenesis precedes gliogenesis. A great deal of evidence from mice in which critical genes have been deleted has shown that glial cell line derived neurotrophic factor (GDNF) (Moore et al., 1996; Pichel et al., 1996; Senchez et al., 1996) and its signaling receptors, the GPI-anchored binding receptor, GFRα-1 (Cacalano et al., 1998) and its transducing receptor tyrosine kinase, Ret, (Schuchardt et al., 1994) are essential for normal development of the entire ENS distal to the esophagus and immediately adjacent cardiac stomach. The GDNF/GFRα-1/Ret signaling components promote proliferation, migration, survival and differentiation of ENCDC (Chalazonitis et al., 1998a; Gershon, 2010; Heuckeroth et al., 1998; Young et al., 2001, Young and Newgreen, 2001). Consequently, mutations in the genes encoding any of the molecules involved in GDNF signaling can result in aganglionosis of the bowel. Congenital aganglionosis or Hirschsprung's disease (HSCR) in humans is usually associated with a hypomorphic mutation in the coding region, or in a conserved enhancer sequence of the first intron of *RET*, although many other genes have also been linked to sporadic or syndromic cases of HSCR (Amiel et al., 2008; Hofstra et al., 2000; Seri et al., 1997). Because the length of gut that is aganglionic in HSCR is highly variable and the penetrance of HSCR is incomplete, it is likely that modifier genes work together with hypomorphic *RET* mutations to influence the ultimate outcome (Owens et al., 2005).

Signaling molecules in addition to GDNF are also expressed in the microenvironment of the fetal gut and most of these have been demonstrated to act later than GDNF to promote differentiation of subsets of enteric neurons. Such factors also have the potential to regulate cell survival and death in the developing ENS. Neurturin and its binding receptor GFRα-2,

for example, which like GDNF activate Ret, also play roles in ENS development (Heuckeroth et al, 1998). Endothelin 3 (ET3; Edn3) is produced in the enteric mesenchyme, stimulates its preferred receptor, endothelin B (ET-B; Ednrb), and plays a critical role in enabling ENCDC to colonize the bowel (Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994). ET-3/Ednrb signaling (and expression of the essential transcription factor, Sox10) synergizes with that of GDNF/Ret (Barlow et al., 2003;Carrasquillo et al., 2002; McCallion et al, 2003; Stanchina et al., 2006). The region of maximal GDNF expression in the enteric mesenchyme moves proximo-distally as a function of developmental time and remains ahead of the advancing front of vagal ENCDC. Because GDNF attracts ENCDC, it is possible that the GDNF gradient is important in leading the advance of ENCDC down the gut. GDNF expression, however, peaks in the cecum. That means that it is necessary to break the GDNF-ENCDC attraction at that point, to prevent the migration of ENCDC from stalling in what would be a cecal trap. ET-3 opposes the GDNF-ENCDC attraction and this effect is likely to be critical in enabling ENCDC to get beyond the cecum, to carry on and complete the colonization of the hindgut. ET-3/Ednrb signaling also appears to be necessary to inhibit the premature differentiation of ENCDC into neurons (Druckenbrod and Epstein 2009; Wu et al., 1999; Gershon, 2010), which again enables the ENCDC population to continue to migrate into the terminal bowel. Mutations in *EDNRB*, therefore, as well as those in *RET*, contribute to the pathogenesis of HSCR in a subset of patients (Puffenberger et al., 1994).

Additional factors of the enteric microenvironment that influence ENS development and that appear to affect ENS cell survival and death include the neurotrophin, NT-3, (Chalazonitis et al., 1994, 2001) and the neuropoietic cytokines, CNTF and LIF (Chalazonitis et al., 1998, a, b). NT-3, CNTF and LIF, unlike GDNF, promote *in vitro* differentiation of enteric glia as well as neurons. Furthermore the neuregulin NRG-1/GGF-2 is expressed in the gut from mid to late gestation and promotes proliferation and survival of enteric glia by stimulating ErbB3/ErbB2 receptors (Chalazonitis et al., 2011a).

Recently the bone morphogenetic proteins BMP-2 and -4 have been demonstrated to play critical roles in differentiation of both neuronal and glial enteric precursors. These proteins influence the migration of ENS precursors along the length of the fetal gut (Fu et al., 2006; Goldstein et al., 2005), promote the dependence of enteric neurons and glia on other neurotrophic factors for survival through upregulation of the corresponding receptors (Chalazonitis et al., 2004, 2011a), and regulate the aggregation of enteric neurons into ganglia by influencing the polysialylation of N-CAM (Faure et al., 2007; Fu et al., 2006). Evidence from transgenic mice that over express either the BMP antagonist, noggin, or BMP-4 suggests that BMPs regulate not only the numbers and density of enteric neurons and glia, but also the relative proportions of different types of neurons in the ENS and the glia to neuron ratio (Chalazonitis et al., 2004, 2008, 2011a).

Cell death and the developing ENS

Conventional apoptotic cell death of ENCDC or post-mitotic neurons is very uncommon within the fetal gut

Markers for apoptotic cell death have been analyzed in the murine ENS at fetal (E12–E18), post-natal (P0–P14) and adult stages (Gianino et al., 2003). Using antiserum to cleaved caspase-3 as a marker of apoptosis, no surge of immunoreactivity was noted at any time examined. An additional study noted the absence or very rare detection of cleaved caspase-3 positive cells in the E13 ileum of either WT or Ednrb deficient rats (Kruger et al., 2003). Also consistent with the rarity of developmental apoptotic death in the gut, enteric neuronal numbers have been reported to be unchanged from WT in mice that over express BCl-X_L, an anti-apoptotic member of the Bcl2 family (Uesaka and Enomoto, 2010). Moreover, the

numbers of enteric neurons in the ileum and colon of mice that lack the pro-apoptotic members of the Bcl2 family, Bax or Bid, have not been found to differ from those in WT mice (Gianino et al., 2003). All of these observations are consistent with the idea that there is not a great deal of cell death in the fetal ENS. Such findings starkly contrast with the 50% neuronal apoptotic death that occurs in other portions of the central and peripheral nervous systems (Oppenheim, 1991) and with the elimination of development-associated death of sympathetic and other neuron types in Bax deficient mice (Deckwerth et al., 1996; Lindsten et al., 2005). The relative absence of conventional apoptotic death in the fetal ENS is thus quite different from that in other parts of the peripheral and central nervous systems in which apoptotic developmental death is the norm. However, it is important to keep in mind that these findings do not rule out other potential death mechanisms in the fetal ENS.

While static markers such as immunostaining for cleaved caspase-3 have not provided evidence for extensive apoptotic death in the fetal ENS, dynamic imaging of ENCDC in E12.5 murine gut with a Sox10-histone2B transgene reporter has recently permitted detection of low frequency nuclear fragmentation events consistent with apoptosis (Corpening et al., 2011). It thus appears that at least some death of mammalian ENCDC takes place within the gut. This phenomenon has yet to be rigorously quantified to determine how significant its impact is on the total population of enteric neurons and glia, and this should now be feasible using the approach that Corpening et al. (2011) have described.

Despite findings that neuronal cell death appears to be uncommon in the fetal gut, significant levels of neuron death occurs postnatally. Aoki et al. (2007) reported extensive TUNEL labeling of enteric neurons in the proximal colon of P7 mice. Moreover, counts of enteric neurons (identified by staining with cuprolinic blue) in the proximal colon revealed that their numbers decreased by about 2-fold between birth and 4 weeks of age. Aoki et al. (2007) also examined TUNEL labeling in P7 mice null for expression of *Ncx/Hox11L1*, a gene that appears to be involved in the specification of neural lineage among ENCDCs. Such knockout mice develop a megacolon and by 2–6 weeks after birth (but not at birth) have far more neurons in the proximal colon as well as other areas of the ENS compared with WT mice. Because they did not detect increased post-natal birth of ENS neurons in the mutant mice, Aoki et al (2007) suggested that the neuronal hyperplasia was due to a deficiency in cell death. In consonance with this, P7 *Ncx/Hox11L1* knockout mice showed little TUNEL staining in the P7 ENS when compared with their WT controls. Although these findings have yet to be confirmed by others, they do suggest that the ENS, at least in the colon, is subject to post-natal pruning of neuron numbers by a death process that is detectable by TUNEL staining, and that functional abnormalities occur when this death fails to occur.

Apoptotic Cell Death of migrating ENCDC en route to the embryonic gut

Although current studies have not detected a significant amount of apoptotic death of ENCDC within the fetal gut, it has been of interest to determine whether apoptosis occurs in enteric neural precursors *en route* to the fetal bowel. One elegant study has addressed this question in the chick embryo by examining cell death in the neural crest population of the vagal stream (somites levels 1–7) which is the site of origin of most of the cells destined to become ENCDC (Wallace et al., 2009). It was demonstrated that these migrating vagal neural crest-derived precursors undergo apoptotic death that can be detected with the TUNEL procedure or cleaved-caspase-3 immunostaining. In addition, expression of a dominant-negative form of caspase-9 in the neural tube increased the pool of vagal neural crest-cells available to colonize the gut and caused a later hyperganglionosis to occur in the foregut (Wallace et al., 2009). These data thus document that apoptotic cell death does occur within the migrating population of vagal enteric precursors, at least in the chick, and that interference with this death has a significant effect on ENS neuron numbers.

As pointed out in a commentary about these studies in the chick, documentation of comparable findings is needed in developing mammalian systems (Enomoto, 2009). In this regard, a study in the mouse revealed detectable, though sparse, TUNEL labeling in the E9–10 ventro-medial pathway of crest-derived cells migrating to the foregut (Kapur, 1999). This contrasted with the extensive TUNEL labeling of ENS precursors detected in the same pathway in mice homozygous for a dominant mutation in the Sox10 gene (Kapur, 1999). The report of sparse TUNEL labeling of migrating ENS precursors in WT mice needs to be interpreted with caution. On one hand, this could indicate that there is little loss of murine ENCDC on their way to the gut, especially in comparison with a positive mutant control in which essentially all of such cells die. On the other hand, given the rapid death and clearance of cells during development and the multiday migration of precursors to the mouse gut, even a low percentage of TUNEL positive cells at any one time point could be compatible with a significant degree of cell loss. A similar issue of interpretation is posed by the low frequency of ENCDC death within the fetal gut (Corpening et al., 2011). While it thus appears that at least some death of mammalian ENCDC takes place during as well as after migration to the mammalian gut, further quantitative studies are needed to determine whether this degree of death significantly affects the final population of neurons and glia in the ENS.

Taken together then, existing data suggest that normal developmental apoptotic cell death is more significant in ENCDC *en route* to the gut than in their successors within the fetal ENS. Several questions are provoked by these findings. Is the cell death of neural crest-derived cells due to a limiting amount of trophic factor expression along their migratory route? If so, which trophic factor(s) are involved; is it GDNF and do these cells already express Ret? In addition, is this cell death specific to the vagal stream of ENCDC or does it also occur in the sacral stream of ENCDC migrating to the post-umbilical bowel? Finally, how does the magnitude of death of ENCDC migrating to the gut compare with that of neural crest precursors migrating to other areas of the nervous system such as the DRGs or sympathetic ganglia?

Regulation of ENCDC survival within the fetal gut

Because ENCDC apparently undergo rather little apoptotic death within the fetal gut, it is possible that these cells do not require trophic support to survive. Alternatively trophic support might be so abundant within the fetal enteric microenvironment that it is not normally limiting. Should this be so, ENCDC might become subject to apoptotic death if their supporting trophic factor(s) were, under abnormal circumstances, to become deficient. Several types of evidence indicate that ENCDC within the fetal gut do require trophic support and that this is provided at least in part by GDNF. For example, GDNF blocks the rapid death that occurs in E14.5 mouse ENCDC cultured without this factor (Taraviras et al., 1999). In addition, extensive TUNEL staining and cleaved caspase-3 immunoreactivity have been observed in the foregut of E10.5 Ret null mice, but not in WT mice (Taraviras et al., 1999; Uesaka et al., 2008). Consistent with an apoptotic mechanism, such death was rescued by over-expression of the anti-apoptotic protein BCl-X_L (Uesaka and Enomoto, 2010). Moreover, ENCDC survival in culture that is promoted by GDNF is dependent on the successive activation of phosphoinositide 3' kinase (PI3-K) and Akt with the consequent phosphorylation and inactivation of pro-apoptotic FOXO transcription factors (Srinivasan et al., 2005). Taken together, these findings support the view that once ENCDC populate the gut, they do not normally undergo a great deal of developmental death because they are supported by abundant trophic factors including (perhaps exclusively) GDNF. However, if GDNF signaling is impaired, then ENCDC can undergo what appears to be apoptotic death.

The role of GDNF signaling in survival of fetal ENS neurons

As in the case of ENCDC, the apparent paucity of normal apoptotic death of post-mitotic enteric neurons in the fetal gut raises the questions of whether their survival also relies on trophic support and whether they can undergo apoptotic death without trophic signaling. To explore these issues, Uesaka et al., (2007) generated mice in which it was possible to conditionally delete the GDNF receptor GFRα-1 in postmigratory ENCDC. Elimination of GFRα-1 during late gestation resulted in rapid, widespread death of both neurons and glia in the colon and produced an aganglionic phenotype similar to that of Hirschprung's disease. Interestingly, death occurring under these circumstances did not appear to be apoptotic. Few of the dying cells exhibited TUNEL staining or immunostained with antibodies against activated caspase-3 or -7. Moreover, electron microscopic analysis of the dying cells did not reveal nuclear or cytological changes consistent with either apoptotic or necrotic cell death. Additional studies were carried out on cultured colonic ENS neurons that were initially cultured with GDNF and then withdrawn from the factor. GDNF withdrawal triggered death that was characterized by poor TUNEL staining, little detectable activation of caspases-3 and -7, and insensitivity to the caspase inhibitor zVAD-fmk or deletion of the gene encoding the pro-apoptotic protein Bax. These properties are vastly different from those in cultured sympathetic neurons undergoing apoptotic death after NGF withdrawal (Deshmukh and Johnson, 1997; Park et al., 1998).

The evidence just described supports the ideas that at least some enteric neurons do require trophic support during development and that enteric neuron death triggered by loss of this support is non-conventional and is neither apoptotic nor necrotic. One potential clue to the mechanism of cell death in this system is that colonic enteric neuron death in culture that is caused by withdrawal of GDNF is blocked by over-expression of BCl-X_L, a member of the BCL2 family. Although BCl-X_L has well-described anti-apoptotic activity, it also blocks autophagy by binding the autophagy protein Beclin/Atg6 (Pattingre et al., 2005). While there is no direct evidence that autophagy and/or Beclin are required for enteric neuron death after GDNF withdrawal, the rescue effect of BCl-X_L raises the possibility of such involvement.

An important finding made by Uesaka et al. (2007) is that deletion of GFRα-1 from the developing small intestine starting at about E13.5 (just after its colonization by ENCDC) had no effect on cell survival in that part of the gut in contrast to massive cell death in the colon. There was also no effect on survival anywhere in the ENS when GFRα-1 was deleted postnatally. The requirement of GFRα-1 for survival of enteric neurons thus appears to be both spatially and temporally specific. Among questions raised by these experiments are 1) whether factors in addition to GDNF support neuron survival at other times or locations during ENS development, 2) the nature of the mechanism of the cell death that occurs in the colon upon loss of GDNF signaling, and 3) why fetal ENS cells seem to be so resistant to developmental cell death. Also, why are the effects of interference with GDNF signaling so specific to the colon? Is this a reflection of timing and of the degree of maturation of colonic ENCDC cells, which are relatively less mature than those of the small intestine at any given developmental age? Alternatively, do neurons in other areas of the gut survive independently of trophic support or are they supported by trophic factors other than GDNF?

More recent studies have examined survival of fetal enteric neurons in mice in which the GDNF signaling receptor Ret was conditionally inactivated as well as in mice that were hypomorphic for Ret expression (Uesaka et al., 2008; Uesaka and Enomoto, 2010). As in the case of conditional loss of GFRα-1, there was extensive death of neurons exclusively in the fetal colon and this was blocked by BCl-X_L over-expression, but not by loss of Bax. These findings thus support the ideas that colonic neurons depend on GDNF signaling for survival and that loss of GDNF signaling elicits a non-conventional form of death in these cells. Such

observations illuminate in part why the aganglionosis associated with mutations in GDNF/Ret signaling in Hirschprung's disease patients are restricted to the colon. Still to be explained, however, is why colonic neurons appear to be more susceptible to deficient GDNF signaling compared with enteric neurons in other parts of the gut.

As compelling as the Uesaka et al. (2007, 2008) studies appear to be regarding the mechanism by which mouse colonic ENS neurons die when GDNF signaling is suppressed both *in vivo* and *in vitro*, a report by Mwangi et al. (2006) provides a somewhat different view. These workers employed a culture system of enteric neurons derived from the E14.5 rat small and large intestines in which survival was dependent on GDNF (Heuckeroth et al., 1998). Data were presented supporting the model that ENS neurons undergo apoptotic death when deprived of GDNF and that GDNF blocks such death by promoting phosphorylation and inactivation of the pro-apoptotic kinase GSK-3β by a pathway involving activation of PI-3 kinase and Akt. For example, cultured enteric neurons deprived of GDNF or transfected with constitutively active GSK-3β in presence of the factor showed significantly enhanced levels of cleaved caspase-3 immunoreactivity while transfection with an inhibitory dominant-negative form of GSK-3β blocked the cell death induced by GDNF deprivation. In addition, death induced by a PI-3 kinase inhibitor in presence of GDNF was accompanied by an increase in cleaved caspase-3 and of apoptotic nuclei in the cultures. The apparent involvement of PI-3 kinase, Akt, activated GSK-3β cleaved caspase-3 and nuclei with apoptotic morphology in this model system certainly support the idea that interference with GDNF signaling can cause apoptotic death of fetal ENS neurons *in vitro*. Nevertheless the case for an apoptotic mechanism was not fully compelling because it would have been desirable to directly monitor apoptotic nuclei under conditions of GDNF withdrawal alone, as well as to test the effects of caspase inhibitors on death. The reasons for the apparent discrepancy regarding the role of apoptotic death in the rat and mouse GDNF deprivation models remain to be determined. Possible explanations include species differences, different culture systems and the uncertainties inherent in trying to distinguish apoptotic from unconventional mechanisms of death.

Roles for NT-3 and BMPs in survival of ENS neurons

Unraveling the regulation of survival and death of ENCDC and enteric neurons within the intact gut raises a number of challenges. Knockout of key molecules within the entire organism can lead to early lethality, thus preventing analysis of the developing ENS. Moreover, compensatory changes in gene expression can occur in knockout animals, which complicates meaningful data interpretation. Alternative techniques for manipulating the embryonic gut microenvironment have yet to be widely adapted to study of enteric neuronal survival and death. Cell culture has provided a fruitful complementary approach to *in vivo* studies that circumvents some of these problems. Methods have been described for culture of ENCDC as well as of fetal enteric neurons and glia under controlled conditions and this has permitted insight into the roles in ENS development not only of GDNF, but also of neurotrophin-3 (NT-3) and bone morphogenic proteins (BMPs) (Chalazonitis et al., 1994, 2001; 2004). Culture studies have revealed that NT-3 promotes differentiation of a subset of enteric neurons from ENCDC (Chalazonitis et al., 1994, 2001). Underscoring the additional role of NT-3 in promoting survival of such neurons, NT-3 withdrawal for 18–48 hr resulted in a significant increase in the numbers of neurons positive for the NT-3 receptor TrkC that also displayed TUNEL staining. To determine whether nearby non-ENCDC might be a source for NT-3 to support enteric neurons, immunoisolated ENCDC were co-cultured with non-ENCDC from fetal gut mesenchyme. The proportions of TrkC-expressing neurons in such cultures were significantly greater when compared to the same cultures treated with function-blocking antibodies to NT-3 (Chalazonitis et al., 2001). With respect to the question of how dependence on NT-3 is regulated, it was found that pre-exposure of

ENCDC to BMP-2 or -4 promotes precocious TrkC expression. This observation raised the hypothesis that BMPs may promote NT-3 dependence by enteric neurons. To test this idea, ENCDC were allowed to differentiate either with NT-3 alone or with NT-3 plus BMP and were then withdrawn from NT-3 for 16 hr. While about 35% of the neurons treated only with NT-3 underwent death (TUNEL positive) in response to NT-3 withdrawal, this proportion rose to almost 60% for neurons differentiated in presence of both NT-3 and BMP. In contrast neurons exposed both to BMP and NT-3 survived well if they were maintained in the continued presence of NT-3 (Chalazonitis et al., 2004). These observations support the idea that the presence of BMPs during neuronal differentiation of ENCDCs promotes TrkC expression and the consequent dependence of survival on the availability of NT-3.

In line with a possible role for NT-3/TrkC signaling in ENS development *in vivo*, transcripts encoding TrkC are expressed in the E14–E16 fetal rat bowel (Chalazonitis et al., 1994). To determine whether NT-3 supports enteric neurons *in vivo*, the overall densities of enteric neurons and of neuronal subsets of defined phenotype were analyzed in mice that over-express NT-3 driven by the DBH promoter (DBH-NT-3 mice) or in mice lacking either TrkC or NT-3 (Chalazonitis et al., 2001). In the DBH-NT-3 mice, the density of myenteric neurons per ganglion was significantly greater than that of WT littermates. Conversely, in the TrkC and NT-3 KO mice, the densities of neurons in both the myenteric and submucosal plexuses were significantly less than those of WT throughout the gut. In the NT-3 KO mice, neuronal loss was particularly severe (down to 20% of WT) in the submucosal plexus. Because the submucosal plexus develops after the myenteric and is derived from ENCDC that migrate from it (Jiang et al., 2003), the particular dependence of submucosal neurons on NT-3/TrkC signaling is consistent with the possibility that NT-3 selectively affects late-born enteric neurons. This possibility is further supported by the observation that at P22 in TrkC KO mice, the density of CGRP-expressing neurons, a late-born phenotype (Pham et al., 1991) in the submucosal plexus is significantly (~60%) lower than that of WT (Chalazonitis et al., 2001). These observations provide evidence that NT-3 is a survival factor for a late-born subset of fetal enteric neurons and raise the possibility that additional trophic factors may also play such a role.

If NT-3 and/or other trophic factors are required for enteric neuron survival, this raises the question as to why there appears to be so little developmental neuron death in the fetal ENS. One possibility is that the microenvironment of the gut supplies an excess of trophic support that is sufficient to maintain almost all, rather than just a fraction of the total newborn neuron population. The apparent capacity of non-ENCDC fetal gut cells to secrete NT-3 in culture is consistent with this idea.

A role for BMPs and GGF-2 in the survival of enteric glia

TrkC is not the only receptor that BMP-2 and -4 up-regulate in cultures of ENCDC. BMP2 and -4 also upregulate expression of ErbB3, the binding receptor for the gliogenic growth factor, GGF-2, (Chalazonitis et al., 2011a). To test whether BMPs enhance the dependency of enteric glia on GGF-2, just as they do the dependency of neuronal survival on NT-3, cultured ENCDC were permitted to undergo gliogenesis either with GGF-2 alone or with GGF-2 + BMPs, and were then cultured without GGF-2 for additional 2 days and assessed for TUNEL staining. Withdrawal of GGF-2 triggered death of glia that had been exposed only to GGF-2; however twice as many glia died if they had previously been exposed to BMPs plus GGF2. Again, glia survived well, even if they were exposed to GGF-2 plus BMPs as long as GGF-2 was not withdrawn from the culture medium (Chalazonitis et al., 2011a). In parallel with the effects of BMPs on enteric neurons, these experiments indicate that BMPs promote the dependence of enteric glia on a gliotrophic factor. These types of experiment are consistent with the idea that BMPs in the enteric microenvironment enhance

the differentiation of enteric neurons and glia from ENCDCs with the consequent expression by the differentiated cells of receptors such as TrkC and ErbB3, along with acquisition of dependence on the ligands for these receptors.

Several types of evidence suggest that BMPs and GGF-2 regulate survival of glia in the intact fetal ENS. Expression of, and signaling by, ErbB3 is critical for enteric gliogenesis *in vivo* as indicated by observations that mice in which ErbB3 is deleted lack enteric glia (Riethmacher et al., 1997). Moreover in mice that over-express the BMP antagonist noggin, the density of enteric glia is reduced (Chalazonitis et al., 2011a). In contrast, enteric glial density is greater in transgenic mice in which BMP signaling is increased than in WT mice (Chalazonitis et al., 2011a). The expression of BMPs and GGF-2 in the developing bowel is also consistent with a role for each in the survival of developing enteric glia (Chalazonitis et al., 2004, 2011a). Transcripts encoding GGF-2 are expressed in the rat bowel after E14 while the abundance of transcripts encoding BMP-4 surges in late gestation. The levels of transcripts encoding BMP-2 in the gut are also high (10-fold that of BMP-4) throughout development. These expression patterns suggest that BMP-4 and -2 are poised to stimulate responsiveness and dependence of the ErbB3-expressing ENCDC on GGF-2, at the very fetal stages when intense enteric gliogenesis occurs.

Despite the evidence that enteric glia require trophic support for survival, present work has not yet addressed the issue of whether these cells undergo a significant degree of developmental cell death *in vivo.* Because little TUNEL staining and activated caspase-3 immunoreactivity occurs in the fetal ENS, it is likely that glial cell death is no more common than that of neurons. If this is so, the possibility is again raised that the enteric microenvironment secretes a sufficient level of trophic factor(s) to support the entire glial population.

Survival requirements of post-natal enteric neurons

Just as gene deletions or mutations such as those affecting GDNF signaling can lead to death of enteric neurons during fetal development, there are also instances in which gene loss affects neuronal survival only in the post-natal ENS. Recent evidence has demonstrated that when serotonergic signaling via the 5-HT_4 receptor is compromised by knockout of the gene encoding this protein, there is a progressive loss of myenteric neurons in the colon between 1 and 4 months of age (Liu et al., 2009). In WT mice, myenteric neuron density increases by about 20% between birth and 4 months of age and then declines slowly by 12 months. Although myenteric neuronal density in 5-HT_4 receptor KO mice is the same as in WT mice at birth, it does not increase between P0 and 4 months, and there is a steady decline in neuron density after 4 months and by 12 months of age is significantly less (by about 20%) than in WT animals. Interestingly, this loss of neurons in the 5-HT_4KO animals is not accompanied by detectable TUNEL staining. In contrast, autophagy, which is rare in WT myenteric neurons, is prominent in the ENS of 5-HT_4KO mice, both in synaptic specializations and in some nerve cell bodies (Liu et al., 2009). It remains, however, to be seen whether the increased autophagy contributes to death of neurons in this model or whether it might serve to help neurons resist cell death (Sridhar, et al., 2011). Also, the failure to detect TUNEL staining of neurons in 5-HT_4KO mice could reflect the prolonged nature of neurodegeneration in these animals or an unconventional mechanism of death.

In addition to death associated with 5-HT_4 KO, a post-natal decrease in densities of myenteric and submucosal neurons occurs in mice with deletion of the transcriptional regulator HIPK-2 (homeodomain interacting protein kinase-2). Knockout of HIPK-2 leads to a particularly severe loss of the subset of enteric neurons that is dopaminergic and expresses TrkC (Chalazonitis et al., 2011b). The neuronal deficiency in HIPK-2 KO mice increases in

a rostro-caudal gradient from duodenum (no significant effect), jejunum (72% density of WT) ileum (53% of WT) to colon (45% of WT). The enteric neuronal defect also increases as a function of age and is much more severe at P90 than at P1. Interestingly, in the HIPK-2 KO mouse, fetal ENS development proceeds normally; there is no alteration in ENCDC migration or increase in TUNEL staining and the formation of the ganglionic plexuses is apparently normal at birth. Deletion of HIPK-2 also results in death of dopaminergic neurons in the ventral midbrain (Zhang et al., 2007). However, in the CNS, onset of dopaminergic neuron death occurs early, during the period of normal developmental cell death. This observation again stresses the difference between the regulation of cell death in the ENS and other regions of the nervous system.

The mechanisms by which ENS neurons are lost in post-natal HIPK-2 null mice are unclear. Between P7 and P14 when enteric neuron loss becomes significant, there is no accompanying increase in TUNEL staining in the knockout ENS compared to that in WT animals. Between P0 and 1 month of age however, there is an arrest in synaptic maturation, increased immunofluorescence staining for the autophagy marker, microtubule-associated protein1-light chain 3 beta, (LC3B) and an increased incidence of autophagosomes, that, like neuron loss, occurs in an increasing gradient from duodenum to colon (Chalazonitis et al., 2011b). It will be of interest in the future to determine whether neuron loss in this model occurs by conventional or unconventional pathways and whether the observed autophagy, is protective or harmful. HIPK-2 negatively regulates BMP signaling by interfering with activation of specific Smad proteins (Harada et al., 2003). This raises the possibility that deletion of HIPK-2 leads to excessive BMP signaling and an imbalance between BMP and TGF-β signaling. In agreement with this idea, the number of enteric neurons that display nuclear translocation of pSmad 1,5 and 8 is significantly greater in HIPK-2 null than in WT mice, while TGF-β signaling is unchanged. Moreover, the increase in BMP signaling in HIPK-2 KO mice increases from ileum to the colon, similarly to neuron loss (Chalazonitis et al., 2011b). It will be informative to determine in future work whether and how appropriate regulation of BMP signaling is required for long-term survival of enteric dopaminergic neurons as well as why loss of dopaminergic neurons in the ENS caused by knockout of HIPK-2 is more delayed than in the CNS.

The 5-HT_4 and HIPK-2 knockout studies provide compelling evidence that survival of post-mitotic enteric neurons is dependent on active receptor-mediated signaling pathways. The observations that deletion of these genes has no evident effect on cell survival in the ENS during fetal development could reflect a maturation-dependent change in survival requirements or a redundancy/abundance of survival factors in the fetal enteric microenvironment that become relatively scarce in the mature gut.

Concluding remarks

As reviewed here, cell death in the fetal ENS shows both significant similarities and differences when compared with that in other areas of the nervous system. In the embryonic chick, neural crest-derived cells in the vagal migratory stream *en route* to the primordial gut undergo what appears to be conventional apoptotic death (Wallace et al. 2009). Whether and the extent to which this is also true in mammals has yet to be fully investigated. The occurrence of cell death in pre-enteric ENCDC corresponds to the apoptotic death of neuroprogenitor cells observed in other regions of the nervous system. Remarkably however, once the ENCDC enter the enteric microenvironment and proliferate, migrate, and differentiate, current findings indicate that apoptotic cell death becomes uncommon. Although the paucity of cell death in the fetal ENS by apoptotic or other mechanisms needs to be confirmed, it presently appears as though the fetal ENS markedly differs in this respect

from other peripheral ganglia and with many CNS regions in which there is large-scale developmental apoptotic death of differentiated neurons and glia.

Why is there being such an apparent discrepancy between developmental cell death patterns in the ENS and other regions of the nervous system? One possibility is that enteric neurons and glia are unusually resistant to cell death and do not require trophic support as do other neural populations. However, as reviewed here, both *in vitro* and *in vivo* studies indicate that fetal ENCDC, enteric neurons and enteric glia all require trophic factors and die when deprived of such support. A more likely explanation therefore is that the gut microenvironment provides levels of various trophic molecules including (but not limited to) GDNF, NT-3 and GGF2 that is sufficient to support most ENCDC and their successors throughout fetal development. In addition, as the neuronal and glial precursors become post-mitotic and form interconnecting aggregates, their close proximity would permit survival-promoting autocrine and paracrine trophic interactions that would augment support from the fetal gut mesenchyme. This situation is different from that prevailing in many other regions of the nervous system in which the supply of trophic factors appears to be limiting and in which competition for such factors results in developmental elimination of a substantial proportion of proliferating progenitor cells as well as of differentiated neurons and glia.

In many regions of the nervous system, cell death serves to limit size to fit within the confines of the skull and body cavities and to assure that neuron numbers are appropriately matched to their targets (Buss et al., 2006). To achieve these ends, a mechanism has evolved in which excess cells are produced and then eliminated by apoptosis. Why then might have things evolved differently in the ENS? One potential explanation is that ENCDC precursor pool that enters the gut is limited in number and that although ENCDC continue to proliferate within the bowel, the mitotic responses to factors such as GDNF dwindle with increasing fetal age as the ENCDC differentiate into neurons and glia (Chalazonitis et al., 1998a). The number of crest-derived cells that enter the gut has to increase tremendously to provide enough ENCDC to colonize the entire bowel. Moreover, the ENS innervates many structures (including longitudinal and circular smooth muscles) within a vast length of bowel and is also capable, as extra-enteric peripheral ganglia are not, of integrative neuronal activity. It is therefore plausible that there are no precursors, neurons or glia to spare during normal ENS development. Furthermore, retention of a maximal number of cells may help to ensure that the ENS is fully functional at birth when external feeding begins and the bowel becomes essential for nutrition and survival.

The observations reported by Aoki et al. (2007) are intriguing in that they indicate that although substantial neuronal death may not occur in the fetal ENS, it does take place post-natally. If these findings can be confirmed, they would support the idea that the ENS is indeed subject to pruning of neuronal numbers by cell death, but that this process does not substantially commence until after birth. The functional importance of cell death in the ENS is implied by the megacolon that develops in mice lacking Ncx, in which postnatal neuronal loss is deficient and the ENS become hyperplastic (Aoki et al. 2007). The notion that enteric neurons become more vulnerable to cell death after the initial developmental period is supported by the delayed loss of neurons that occurs in mice null for 5-HT_4 or HIPK-2. Perhaps it is only after becoming fully functional that the ENS can afford to lose neurons.

The unique setting of the ENS may also influence cell death levels and mechanisms during and after development. The ENS has a propensity to be subject to traumatic injury since it lacks the bony protection afforded to the CNS and because the contents of the intestinal lumen are septic. This may account, at least in part, for the neuronal loss in the ENS that accompanies ageing. Such loss may be minimized by unusually active neuroprotective mechanisms. In addition, unlike most other areas of the nervous system, neuron loss in the

ENS may be at least partially compensated by replacement from adult stem cells and/or glia (Liu et al., 2009; Laranjeira et al., 2011; Joseph et al., 2011).

When cell death does take place in the ENS, there is the perennial question of whether this occurs by an apoptotic or alternative mechanism. This is not merely an academic issue, especially in the context of Hirschprung's disease associated with malfunction of the GDNF signaling cascade. As discussed above, evidence from mouse models of Hirschprung's disease suggests that an unconventional, non-apoptotic mechanism may mediate the death of colonic ENCDC and mature neurons when GDNF signaling is deficient in vivo (Uesaka et al., 2007). If the molecular nature of this mechanism could be understood, insight might be gained into how to prevent the aganglionosis of Hirschsprung's disease in susceptible individuals. The intriguing observation that over expression of BCl-X_L prevents death of colonic neurons in murine models of Hirschsprung's disease may provide a useful starting point for such investigations.

In summary, current studies have already revealed a great deal about cell death in the developing ENS and how this both resembles and differs from that in other regions of the nervous system. Clearly much more remains to be learned. Understanding mechanisms of cell death in the ENS will not only increase knowledge of how the ENS develops but also about how developmental defects of the ENS might in the future be prevented and how the mature ENS is maintained.

Acknowledgments

Support for the preparation of this review was provided by NIH grants DK58056 (AC), NS033689 (LAG) and NS12969 and NS15547 (MDG).

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Foreword

The authors are honored to contribute to this special issue devoted to the memory of Dr. Marshall Nirenberg. This opportunity is especially meaningful to Drs. Greene and Chalazonitis who were post-doctoral and foreign graduate student trainees, respectively, in Marshall's laboratory at the NIH in the early nineteen seventies.

The enteric nervous system (ENS) is colonized by neural crest derived cells (ENCDC)

Migrating vagal ENCDC en route to the embryonic gut undergo apoptotic cell death

Conventional apoptotic cell death of ENCDC is uncommon within the fetal gut

Abundant levels of growth factors (i.e. GDNF) protect colonic neurons

Post-natal ENS neurons survive through active receptor-mediated signaling pathways