

could represent an end point of the discovery process; for behavioral neuroscience it is the beginning.

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## VIEWPOINT

# Cell death in early neural development: beyond the neurotrophic theory

Enrique J. de la Rosa and Flora de Pablo

**The important effect of cell death on projecting neurons during development is well established. However, this mainstream research might have diverted recognition of the cell death that occurs at earlier stages of neural development, affecting proliferating neural precursor cells and young neuroblasts. In this article, we briefly present observations supporting the occurrence of programmed cell death during early neural development in a regulated fashion that to some extent parallels the death of projecting neurons lacking neurotrophic support. These findings raise new questions, in particular the magnitude and the role of this early neural cell death.**

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THE NEUROTROPHIC theory clearly establishes the role of target-derived neurotrophic factors in the protection of projecting neurons from programmed cell death. This type of cell death is also called naturally-occurring cell death, physiological cell death or apoptosis<sup>1–7</sup>. The neurotrophic theory has provided an essential conceptual basis, not only for the acceptance of cell death as a physiological process in neural development, but also to generalize the significance of cell death during normal development in an organism<sup>8,9</sup>. The concept of

neurotrophism has broadened, and now includes the trophic dependence of different types of neurons, as well as of glial cells, at various developmental stages. Survival signaling relies not only on the classical neurotrophic factors, but also on many other growth factors belonging to several molecular families (including neurotransmitters released by afferent terminals), as well as on the electrical activity of neighboring axons<sup>10–21</sup>. Nevertheless, this expanded perspective has paid little attention to cell death affecting neural precursor cells and young

neuroblasts at early stages of neural development. Consideration of the available evidence on early neural cell death should promote studies of the incidence of this regulated form of cell death and of its role in building the complex cellular organization and function of the nervous system.

### Early neural cell death affects proliferating neural precursor cells and young postmitotic neuroblasts

A regulated balance between cell proliferation and cell death is, in many developmental systems, an appealing mechanism to determine the size and the cytoarchitecture of tissues, organs and organisms<sup>9,22–24</sup>. Although coordination is considered necessary among the main cellular processes in neurogenesis<sup>25</sup> (i.e. proliferation, migration, differentiation and cell death), the effect of cell death on proliferating or recently differentiated cells has largely been ignored. This is surprising, because the natural incidence of cell death in the early embryo neuroepithelium was described in the seminal work of Glücksmann in 1951 (Ref. 26). Few neurons, if any, are differentiated at these developmental stages, therefore the dead cells must be proliferating neural precursor cells or recent postmitotic cells, not neurons connected with their targets. Unfortunately, most of the observations to date, some of which are summarized in Table 1, do not provide a thorough identification of the dead cells, in spite of the existence of early studies that move towards cell characterization<sup>47,51</sup>. In a few cases where the dead cells were characterized at early developmental stages, most apoptotic cells were identified as having been recently engaged in the cell cycle (based on their capacity to synthesize DNA shortly before death)<sup>32,36,39,45,47</sup> (Fig. 1).

Recent knockout mouse studies have provided dramatic proof of the occurrence of cell death during early neural vertebrate development; inactivation of several regulatory or executor molecules in the apoptotic pathway caused embryonic or perinatal lethality<sup>52–54</sup>. Unexpectedly, the CNS was the most affected tissue, presenting a large excess of neurons in disorganized structures. Interestingly, morphological defects were already visible well before neuronal generation, as early as embryonic day (E) 9.5 in the null mutant for the apoptotic activator Apaf-1 (Ref. 54), and at E10.5 in the null mutants for caspase 9 (Ref. 53) and caspase 3 (Ref. 52). These observations unequivocally support the necessity of neural precursor cell death in the normal neural development in vertebrates. Identification of dead cells in normal development or, conversely, of the excess of surviving neural cells in models of knockout mice, as either proliferating-neuroepithelial cells or young neuroblasts is possible<sup>32,36,39,45,47</sup> and should be performed routinely.

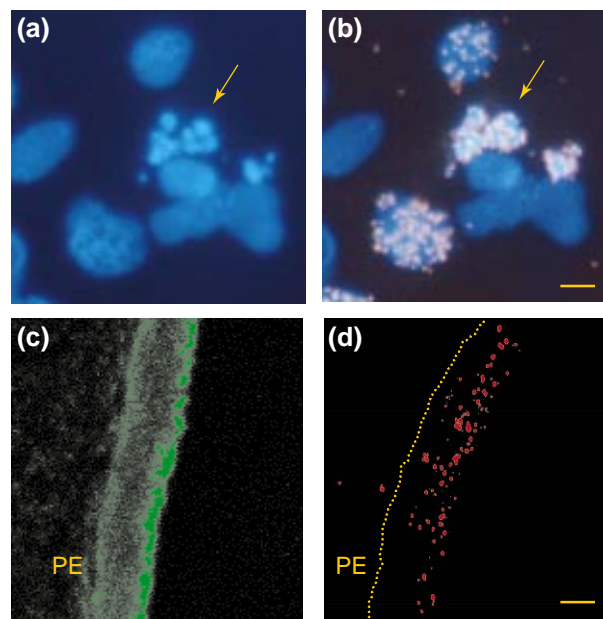
### Is early neural cell death a significant process?

Determination of cell death numbers is an important piece of the puzzle for assessing the physiological relevance of this process, as well as for the accurate interpretation of observations such as lineage analysis, or proliferative and differentiative effects of signals<sup>15,55</sup>. For example, some of the effects of growth factors, originally interpreted as stimulation of proliferation, might be the result of inhibition of cell death. This possibility has not been analyzed

**TABLE 1. Localization of early neural cell death in vertebrates**

Developmental process	Location	Refs
Neurulation	Neural plate, neural fold and neural tube	26–29
Neural crest formation	Premigratory and migratory neural crest cells	30–33
Eye induction and formation	Forebrain, optic vesicle and optic cup	26
Neurogenesis	Neural tube and spinal cord	29,32,34,35
Neurogenesis	CNS	36
Neurogenesis	Cerebral cortex	37–39
Neurogenesis	Retina	40–46
Neurogenesis	PNS ganglia	26,47–50

because early neural cell death has previously been ignored or assumed to be insignificant. In addition, determination of the magnitude of neural precursor cell death is not an easy task. The impact of target-derived neurotrophic competition in the reduction of neuron numbers has been evaluated by tracking the reduction of a differentiated population as development proceeds and in rescue experiments by



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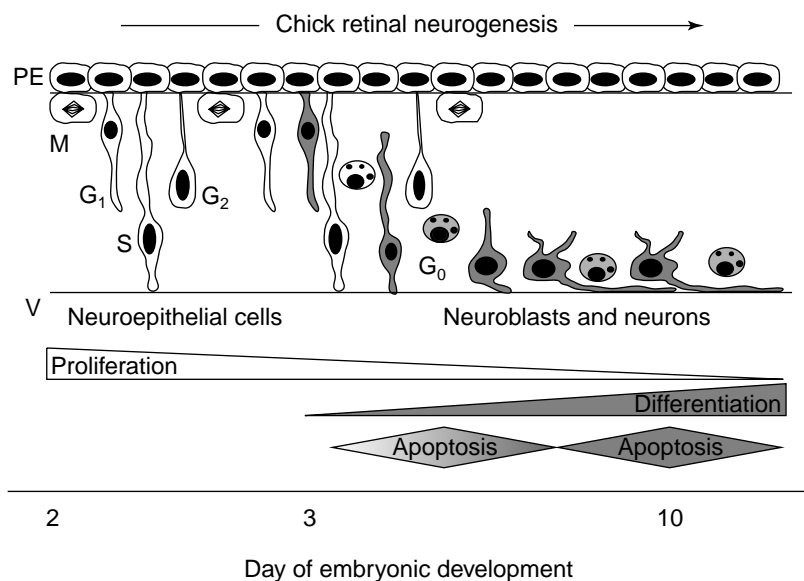
**Fig. 1. Early cell death in the embryonic chick retina prominently affects cells other than neurons.** (a) and (b) Embryonic day (E) 5 neuroretinas were cultured for 6 hrs in the presence of [methyl-<sup>3</sup>H]thymidine, then processed as dissociated cells for DAPI staining (blue) to characterize dead cells [the arrow in (a) indicates a pyknotic nucleus]. The same preparation had been processed for autoradiography to develop [methyl-<sup>3</sup>H]thymidine labeling present in pyknotic nuclei, and in normal nuclei (b). (c) and (d) Cryosections of E4 neuroretinas stained for Islet-1/2 [shown in green in (c)], a marker of differentiated ganglion cells, and by TUNEL [terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end-labeling] [shown in red in (d) a serial section with a similar field to (c)] to localize the dead cells. The TUNEL-positive cells are more broadly distributed than the Islet-1/2-positive cells, the only postmitotic neurons at this stage. The pigmented epithelium (PE) is labeled with a broken line in (d). Scale bar, 2  $\mu$ m in (a) and (b), and 40  $\mu$ m in (c) and (d). Reproduced, with permission, from Refs 45 and 46.

administration of survival factors<sup>56–58</sup>. A similar approach cannot be used to quantify the incidence of programmed cell death in early neurogenesis, because proliferation, differentiation and apoptosis coexist. Dead cells are studied at isolated time points in development, routinely by visualization of late apoptotic stages after staining of pyknotic nuclei or by labeling of DNA breaks using TUNEL [terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end-labeling] or ISEL+ (*in situ* end labeling). Dead cells are normally found in low proportions, both in the few available quantitative studies<sup>45,46</sup> and in the more frequent non-quantitative observations (Table 1). These observations parallel studies at later developmental stages, in which reported dead cell numbers are low in spite of the fact that defined neuronal and glial populations are halved as a result of cell death<sup>9,11</sup>. This is illustrated in oligodendrocyte generation in the optic nerve, where the maximum proportion of pyknotic nuclei found is 0.30%, in spite of the estimated 50% accumulative cell death during development<sup>11,59</sup>. Similarly, in the retina there is 51% total neuronal cell death in the ganglion cell layer and 46% in the inner nuclear layer but these are visualized as only 0.33% and 0.46% pyknotic nuclei, respectively<sup>55</sup>. These values are in the range for the proportion of pyknotic nuclei (~0.20%) found for early neural cell death in the retina<sup>45,46</sup>. Nonetheless, it is misleading to extrapolate the true relevance of the neural cell death process from the actual numbers of dead cells found

because apoptotic cells are rapidly removed by their neighbors in an estimated time of a few hours or less<sup>15,36</sup>. Using the ISEL+ technique Blaschke *et al.*<sup>36,38</sup> performed a thorough analysis of cell death in the mammalian brain. However, the high proportion of labeled cells (over 50%) found at each particular time-point analyzed is not easy to harmonize with the growth of the nervous system during embryogenesis. Thus, additional studies in different regions of the developing nervous system, at various developmental stages, are required to ascertain the validity of the method and the generality of the observations. In addition, the results should be re-evaluated in terms of additional sources of DNA-breaks<sup>60,61</sup>. Estimations based on the neurogenesis rate with respect to cell-cycle time also suggest that 50–70% of total neural precursor cells might die during proliferation<sup>62</sup>. Although all the available extrapolations have shown that the magnitude of early neural precursor cell death is similar to that of late neuronal cell death, more direct quantitative experiments need to be carried out. Earlier markers of the commitment to cell death as well as specific markers of neural precursor cells should assist in this direction. Determination of neural cell types and numbers in the early developmental stages of the knockout mice for the apoptotic pathway showing neural hyperplasia, should confirm the magnitude of early neural cell death.

#### Cell survival and death are precisely regulated processes in early neural development

Recognition of cell death in early stages of neural development has been slow, but identification of the signals that control it is lagging even more. However, the few available examples suggest that survival is regulated by limiting amounts of locally produced growth factors: the products of the same cell or neighboring cells. Furthermore, progression of the cells through neurogenesis appears to require a coordinated, sequential action of various survival signals acting in an integrated network<sup>9,16</sup>. The chick embryo neuroretina, a relatively well characterized structure in this respect, presents defined patterns of death, termed isothanas<sup>45</sup>. In this system, two members of the insulin family sequentially promote neurogenesis, first proinsulin and later insulin-like growth factor-1 (IGF-I) (Refs 45,46,63,64). These factors mediate their actions on proliferation and differentiation, at least in part, by a primary decrease in apoptosis, affecting neuroepithelial cells and recently differentiated neuroblasts<sup>45,46</sup>. Brain-derived neurotrophic factor (BDNF) acts subsequently to promote survival of young-retinal neuroblasts<sup>44</sup> and, finally, neurotrophin-3 (NT-3) controls retinal ganglion cell numbers<sup>65,66</sup>. Altogether these results suggest the existence of at least two distinct waves of cell death in neurogenesis for the chick embryonic retina (Fig. 2). The scenario of an integrated network of growth factors and other extracellular signals maintaining early neural cells is also supported by observations in motoneuron development. An early phase of young motoneuron cell death, unresponsive to all the tested factors, is followed by a later phase of neuronal cell death responsive to trophic support<sup>34</sup>. Neural crest development also provides several examples of coordinated growth factor support, including concerted



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**Fig. 2. Early cell death is a genuine process of neurogenesis.** Early neurogenesis in the embryonic chick retina, framing the phases of neural precursor cell death and ganglion cell death. Proliferating neuroepithelial cells (white cells cycling throughout M, G<sub>1</sub>, S, G<sub>2</sub> and M phases) leave the cell-cycle to become neuroblasts and neurons (gray cells in G<sub>0</sub>, migrating to their definitive layer; for simplicity only ganglion cells have been depicted, although other neuronal and glial cell types are generated during the period shown). The proliferation of neuroepithelial cells decreases as neurogenesis proceeds, while neuronal and glial differentiation increases; both processes coexist with one another, as well as with cell death. Classical neuronal cell death affects differentiated ganglion cells with a peak at approximately embryonic day (E) 10. An earlier, distinct phase of apoptosis occurs with a peak at approximately E5, and affects cells other than fully differentiated neurons. This early phase could include different, partially overlapping types of cell death<sup>34,44,53</sup>. Abbreviations: PE, pigmented epithelium; V, vitreal surface.

action by stem cell factor (SCF), fibroblast growth factor 2 (FGF-2), BDNF and NT-3 (Refs 33,67), by IGFs, FGFs and NT-3 (Refs 33,68), and by neuregulin and NT-3 (Ref. 48). Additional studies, in different parts of the nervous system, using comparable techniques are required for an integrated view of the different phases of cell death and of the signals promoting survival. Moreover, early neural cell death can be provoked by death-factor signaling. The BMP (bone morphogenetic protein) signal results in neural crest cell depletion in specific locations<sup>31</sup> and the NGF signal results in retinal cell death through the p75 receptor<sup>43</sup>. Both cell survival and death are likely to be mediated by apoptotic pathways, which have been characterized in other systems<sup>69,70</sup>. However, there has been little investigation of the defined pathways that act in early neural cell death in vertebrates.

In addition to exogenous signaling, asymmetries intrinsic to the neuroepithelial cells might also be relevant in determining the susceptibility of cells to death<sup>15</sup>. This aspect is poorly understood and would include asymmetric cell division<sup>71</sup>, scattered Delta expression associated with lateral inhibition of neuronal differentiation<sup>72</sup> and the dynamic, restricted expression of the chaperone heat shock cognate 70 (Hsc70) (Refs 73,74) of known antiapoptotic activity. Depending on the expression of these cell autonomous determinants, some cell populations might be more death-prone.

### What is the role of cell death in early neurogenesis?

Data presented to date are suggestive of an important role for early neural cell death in neural development. This is specially supported by the dramatic phenotype observed when death is lacking, as a consequence of caspase 3, caspase 9 and Apaf1 gene disruption<sup>52–54</sup>. However, the precise biological function of developmental cell death, including that affecting differentiated neurons remains largely speculative<sup>75,76</sup>. Indeed, in the pioneering *C. elegans* model, animals with reduced cell death and thus, with extra neurons, present no obvious dysfunctional phenotype<sup>77,78</sup>. Multiple roles for developmental cell death in neurogenesis are possible in principle, as have been postulated for developmental cell death in general<sup>18,15,26,75</sup>. These include morphogenesis, sharpening of compartment limits, selecting fitness by eliminating abnormally located or differentiated cells and controlling cell numbers. A possible specific role might be closely related to the central point of neurogenesis, this is the commitment to leave the cell cycle and differentiate, or to continue proliferating with subsequent phenotypic selection<sup>32,36,45,79</sup>. Detailed analysis of genetically modified models should clarify the role of cell death in neural development, as in the case of *Drosophila*. In this model, cell death is considered a final cell fate contributing to retinal patterning<sup>80</sup>. An in-depth analysis of the apoptotic pathway in knockout mice might also answer why, and how many, cells have to die to achieve normal neural development. In addition to the possibilities already mentioned, the intriguing implication of somatic gene recombination and subsequent cellular selection by apoptosis in the generation of neuronal diversity has recently been postulated<sup>60,61,81</sup>.

Knockout mice for the molecules responsible for repairing double-strand DNA breaks caused during somatic recombination of B-lymphocyte immunoglobulin or T-cell receptor genes, showed increased early neural cell death that was already visible at E10.5 (Refs 82,83), illustrating at least one of the possible reasons why a cell might die.

### Concluding remarks

Although we are still far from a coherent, integrated view, the evidence presented supports the relevance of early neural cell death in nervous system development. Careful analysis at early stages of neural development of the available knockout mice, showing alterations in neuronal or neural precursor cell numbers, should validate the viewpoint formulated here. In addition, complementary approaches to modify gene expression, such as retroviral gene transfer or interference with mRNA, have started to provide powerful tools for dissecting the process of early neural cell death. To extend and adapt the neurotrophic concept to early neural development will require a lot of effort in multiple model systems, but it will be as fascinating a task as when the theory was originally elaborated for projecting-neuron cell death.

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