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Revisión

Early neural cell death: an overlooked process in neural development

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ABSTRACT

During the development of the vertebrate nervous system, multiple physiological processes are involved in the generation of its complex cytoarchitecture and functionality. Among them, programmed cell death has been recognized as a key process that affects connecting neurons. By contrast, there is limited information available regarding the cell death that affects neuroepithelial cells, and recently born neurons and glia, hindering the comprehensive understanding of neural development. We have demonstrated that exquisitely regulated PCD occurs during early stages of neural development such as neurulation and neurogenesis. We have characterized how survival signals from proteins like proinsulin/insulin, c-Raf, and HSC70 counteract caspase-dependent apoptosis, which affects neuroepithelial cells proliferation and the generation of retinal ganglion cells. Furthermore, the

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characterization of these physiological signals during retinal neurogenesis has the potential to provide new therapeutic tools to attenuate retinal neurodegeneration.

Key words: Programmed cell death, apoptosis, neurulation, neurogenesis, proliferation, differentiation, retina, proinsulin, insulin, c-Raf, HSC70, caspases, neuroprotection, neurodegeneration.

RESUMEN

Muerte neural temprana: un proceso inadvertido en el desarrollo del sistema nervioso.

Durante el desarrollo del sistema nervioso de vertebrados, múltiples procesos fisiológicos participan en la generación de su compleja arquitectura celular y funcionalidad. Entre ellos, la muerte celular programada que afecta a neuronas de conexión está reconocido como un proceso fundamental. Por otro lado, hay escasa información disponible acerca de la muerte celular que afecta a células neuroepiteliales y a neuronas y glía recién nacidas, lo que impide que tengamos una noción completa sobre el desarrollo neural. Los estudios de nuestro laboratorio han demostrado que la muerte celular programada se encuentra finamente regulada y ocurre en etapas tan tempranas del desarrollo como la neurulación o la neurogénesis. Hemos caracterizado el papel que moléculas de supervivencia, como la proinsulina/insulina, c-Raf o HSC70, desempeñan bloqueando la apoptosis dependiente de caspasas, proceso que afecta a células neuroepiteliales proliferativas, así como a la generación de las células ganglionares de la retina. Es más, la caracterización de estas señales fisiológicas originadas durante la neurogénesis de la retina nos ha proporcionado una nueva herramienta terapéutica potencial para el tratamiento y atenuación de las neurodegeneraciones retinianas.

Palabras clave: Muerte celular programada, apoptosis, neurulación, neurogénesis, proliferación, diferenciación, retina, proinsulina, insulina, c-Raf, HSC70, caspasas, neuroprotección, neurodegeneración.

INTRODUCTION

The nervous system is a sophisticated structure sustained by a complex collection of cell types and stereotypic connections. Through proliferation and differentiation, as well as cell migration, axonogenesis and dendritogenesis, undifferentiated neuroepithelial cells become highly differentiated neurons or glia, and they establish an intricate network of connections. Programmed cell death (PCD) affects both projecting neurons and other types of differentiated

neurons and glia, and it has been clearly recognized as a fundamental element in generating and refining the complexity in the nervous system. For example, differentiated neurons that do not succeed in establishing the appropriate synaptic connections or that show impaired electrical activity are selectively eliminated by PCD in order to yield a highly specialised functional network. At this stage, the death/survival decision in neurons is tightly regulated and depends on the availability of neurotrophic factors as well as on cell-cell interactions (1-6).

In striking contrast to the extensively studied cell death of mature neurons, little attention has been paid to the PCD that affects proliferating neuroepithelial cells and recently born neuroblasts (7-9). For the last 12 years, we have studied this early phase of cell death in the developing nervous system, characterizing the cell populations affected, the regulatory mechanisms involved, its magnitude and the functional implications of this process. In the present review, we shall summarize all these aspects of early neural cell death, emphasizing that our limited knowledge of this process may be hindering the integrated understanding of neural development and vindicating the more extensive study of this process.

CELL DEATH OCCURS DURING EARLY STAGES OF NEURAL DEVELOPMENT

The nervous system mostly derives from the neural tube, a structure generated from the embryonic ectoderm through inductive interactions and morphogenic movements. Since the seminal work of Glücksmann (10; see 7 for additional references), apoptotic cells have been clearly identified during neural induction and neurulation in vertebrates. In the neurulating chick embryo, apoptotic cells can be observed when the neural tube is forming and regional specification is taking place. Indeed, TdT-mediated dUTP nick-end labelling (TUNEL) of fragmented DNA, a hallmark of apoptosis, reveals dying cells in precise locations of the embryo, for instance at the anterior neuropore, the dorsal part of some prosomeres and rhombomeres, the presumptive anlage of the otic vesicle (Figure 1, A-D).

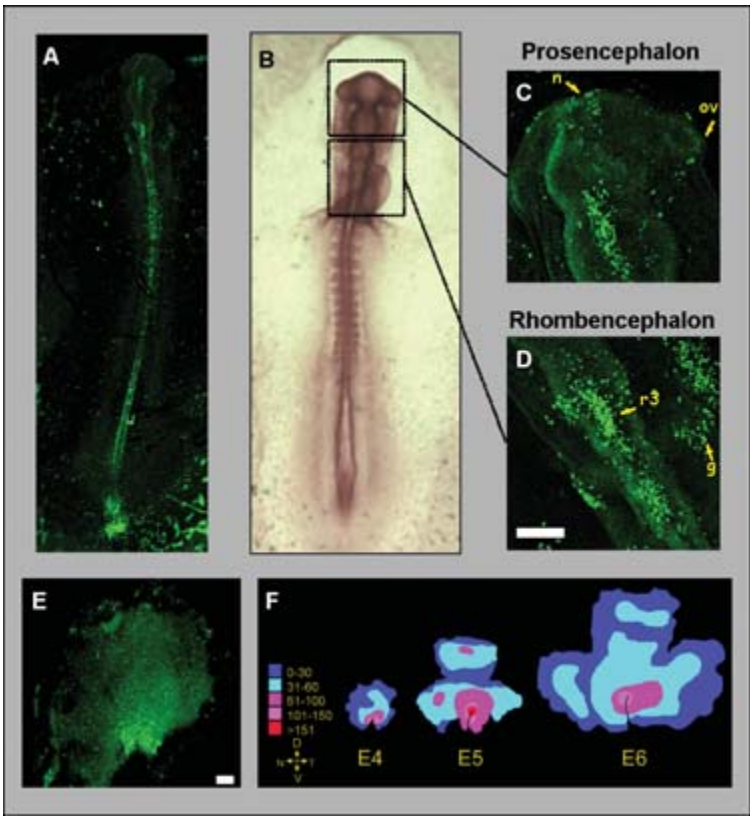

FIGURE 1. ***Distribution of cell death during early neural development in chick embryos***. *Apoptotic cells were identified by TUNEL of freshly dissected neurulating embryos (A, C, and D) or of the embryonic retina (E). The whole embryo image (A) was captured with a 2.5 × objective on a confocal microscope. Serial images (z-axis) were captured every 10 μm with a 10 × objective at the levels indicated by the insets in (B) and compiled (C, D). The main morphological features are labeled: g, presumptive otic vesicle anlage; n, anterior neuropore; ov, optic vesicle; r3, rhombomere 3 [reproduced with permission from (23); © 2002 Federation of European Neuroscience Societies]. Whole-mount E4 retina visualized with a 2.5× objective on a confocal microscope (E). The density and distribution of TUNEL positive nuclei in E4, E5 and E6 retinas is represented as isothanas depicted from labels retinas as that shown in E (F). The arbitrary pseudocolor scale corresponds to TUNEL-stained apoptotic bodies per square millimetre. The color scale represents pyknotic bodies per microscopic field (0.18 mm²). The orientation of the retinas is indicated: N, nasal; T, temporal; D, dorsal; and V, ventral. The black line represents the optic nerve fissure [reproduced with permission from (35); © 1999 Federation of European Neuroscience Societies] Calibration bar, 0.5 μm (A-B), 250 μm (C-D) and 0.4 μm (E).*

We have also characterized the process of cell death in the chick and mouse neuroretina, a classic model system in developmental neurobiology (11-14). Apoptotic cells appear throughout the development of the retina, from the early proliferative to the late synaptogenic stages (15, 16; see 17 for additional references.). Even at early developmental stages when neurogenesis begins, distinctive and prominent patterns of cell death can be seen, following the centro-peripheral gradient that parallels that of differentiation (18) (Figure 1, E-F).

EARLY NEURAL CELL DEATH AFFECTS BOTH PROLIFERATING NEUROEPITHELIAL CELLS AND RECENTLY BORN NEUROBLASTS

The presence of dying cells during early stages of neural development, neurulation and neurogenesis, implies that cells other than connecting neurons are affected since such connections are not yet established. Unfortunately, these dead cells have not been specifically identified in most studies of normal or manipulated embryos (see 7 and 9 for additional references). In the embryonic chick retina, we have identified dead cells as having recently exited S-phase of the cell cycle, since they could incorporate labelled DNA-precursors shortly before displaying apoptotic phenotype. In addition, some apoptotic cells express early neuronal markers (Figure 2).

EARLY NEURAL CELL DEATH IS A PRECISELY REGULATED PROCESS

The physiological relevance of any biological process can be confirmed by determining the mechanisms that regulate it, which also provides useful tools to define the magnitude and function, in our case of early neural cell death. Interfering with cell death at early stages produces abnormal development, as demonstrated by embryonic and genetic manipulation. Knockout-mouse studies have not only provided cues regarding the regulation of cell death but also, dramatic proof that cell death occurs during early neural

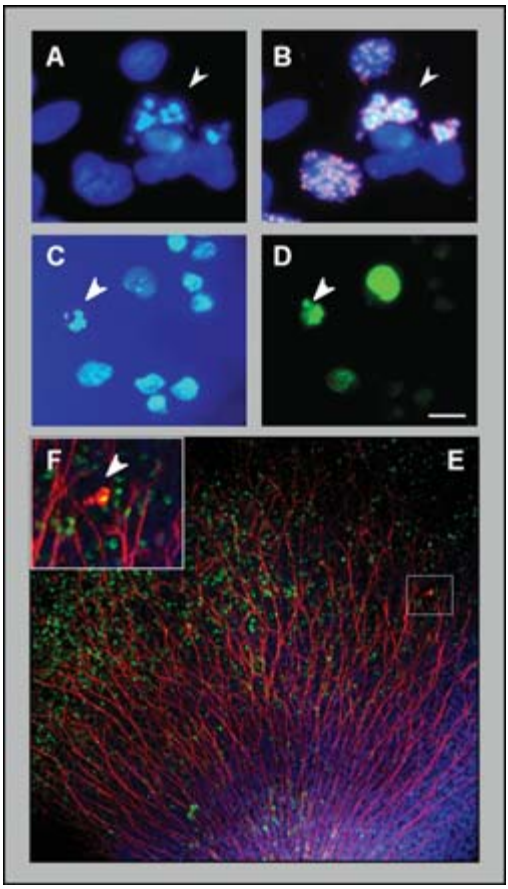

FIGURE 2. ***Characterization of the cells affected by early neural cell death.*** *E5 chick retinas were dissociated and stained with DAPI to visualize the nuclear morphology (blue in A-C). Islet 1/2 immunostaining was performed to identify recently differentiated neurons (green in D, same field as in C). E5 retinas were labeled with [^{3}H]-thymidine, dissociated and processed for autoradiography to detect proliferating cells (grains in B, same field as in A). Arrowheads indicate the pyknotic nuclei of apoptotic cells [reproduced with permission from (35); © 1999 Federation of European Neuroscience Societies and from (20); © 2000 The Company of Biologists Ltd.]. Whole-mount E5 chick retinas were triple-stained with DAPI (blue), for BrdU (green) and for TUJ1 (red). Superimposed confocal microscope images illustrate the distribution of differentiated (TUJ1-positive) and proliferative (BrdU-positive) cells (E). (F) corresponds to higher magnification field of the inset in (E), where an apoptotic, BrdU-positive and TUJ1-positive cell can be observed (arrowhead). Calibration bar, 5 μm (A-D), 50 μm (E) and 10 μm (F).*

vertebrate development. Genetic inactivation of both proapoptotic and prosurvival regulatory molecules, as well as apoptotic executor molecules, causes embryonic or perinatal lethality associated with severe malformations that mostly affects the nervous system (see 9 for a detailed review). Interestingly, morphological defects were already visible at early stages of neural development, concomitant with neurulation and the first waves of neurogenesis.

Embryonic manipulation in the chick is a classic approach that has been employed to characterize how cell death is regulated (19-24). We have focused on survival factors as attenuators of early neural cell death, and in particular proinsulin/insulin (25). *In ovo* treatment of chick embryos with insulin-receptor blocking antibodies (20), or with antisense oligonucleotides (ODNs) (21), augments apoptotic cell death (Figure 3, A-B). In the retina, the increase in apoptosis parallels a decrease in the number of retinal ganglion cells (RGCs) (Figure 3, C-D). Conversely, *in ovo* treatment with proinsulin reduces naturally occurring cell death but induces embryonic malformations, including asymmetric closure of the neuropore, collapse of the optic vesicles, and bending of the neural tube (Figure 4).

We have further characterized the putative signalling pathway downstream of proinsulin/insulin at different levels. Upon binding to its tyrosine-kinase, membrane-bound receptor, proinsulin/insulin activates both the PI3K/Akt and the Ras/Raf/MAP kinase signaling cascades. Significantly, RGC survival is regulated by c-Raf in the embryonic chick neuroretina (26) and retroviral transfection of a dominant negative form of c-Raf increases PCD while reducing the density of RGCs correctly situated in their layer (Figure 5). Furthermore, proinsulin/insulin upregulates the expression of HSC70, a chaperone involved in survival under conditions of cell stress. Blocking proinsulin expression with antisense ODNs in cultured neurulating chick embryos diminishes HSC70 expression and increases cell death (27). Indeed, antisense ODN interference experiments established a correlation between proinsulin-induced cell survival and HSC70 expression, both in cultured chick embryos and *in ovo* (23). Thus, HSC70 silencing increases caspase-3 activation and apoptosis in those embryonic regions where PCD was already naturally ongoing (Figure 6).

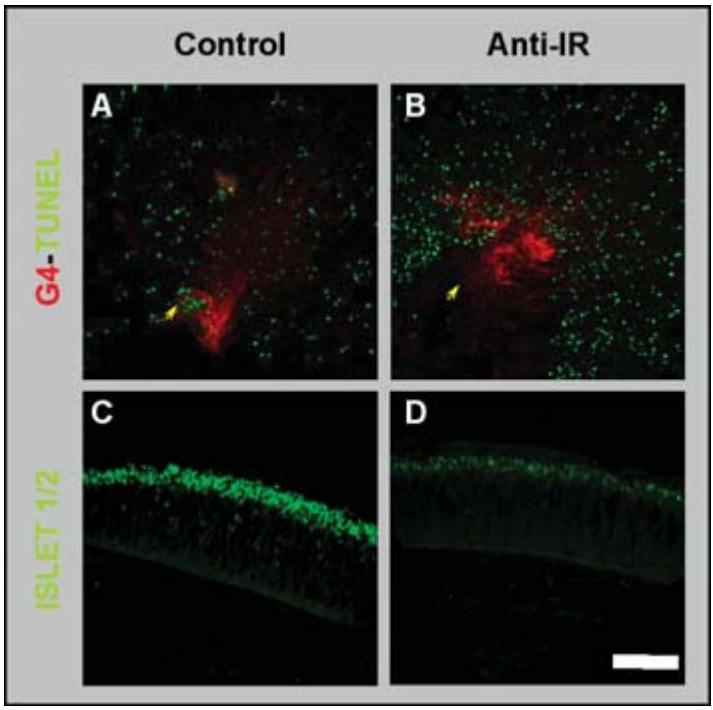

FIGURE 3. ***Interference with proinsulin/insulin signaling causes cell death in the embryonic chick retina.*** *E2 chick embryos were treated in ovo for 2 days (A, B) or for 4 days (C, D) with anti-insulin receptor Igs (Anti-IR) or the corresponding control Igs. The neuroretinas were then processed in whole-mount (A, B) or as cryosections (C, D). TUNEL (green in A and B) was employed to characterize the apoptotic cells. Immunostaining for G4/Ng-CAM (red in A, B) or for Islet 1/2 (green in C, D) was employed to identify differentiated ganglion cells. Superimposed confocal microscope images illustrate the distribution of the dead cells surrounding the optic nerve head and the retinal ganglion cell axons (A, B). Fields adjacent to the optic nerve in retinal sections show the effects of treatments on Islet1/2-positive cells [C, D: reproduced with permission from (20); © 2000 The Company of Biologists Ltd.]. Calibration bar, 50 μm.*

Caspases have traditionally been considered the main executioners in PCD (28) and their involvement in early neural cell death is supported by the phenotypes of caspase knock-out mice (29, 30, see 9 for additional references). Experimental manipulation of the chick embryo has confirmed that caspases are involved in early neural cell death (23, 24). Thus, short-term treatment of chick embryos i*n ovo*

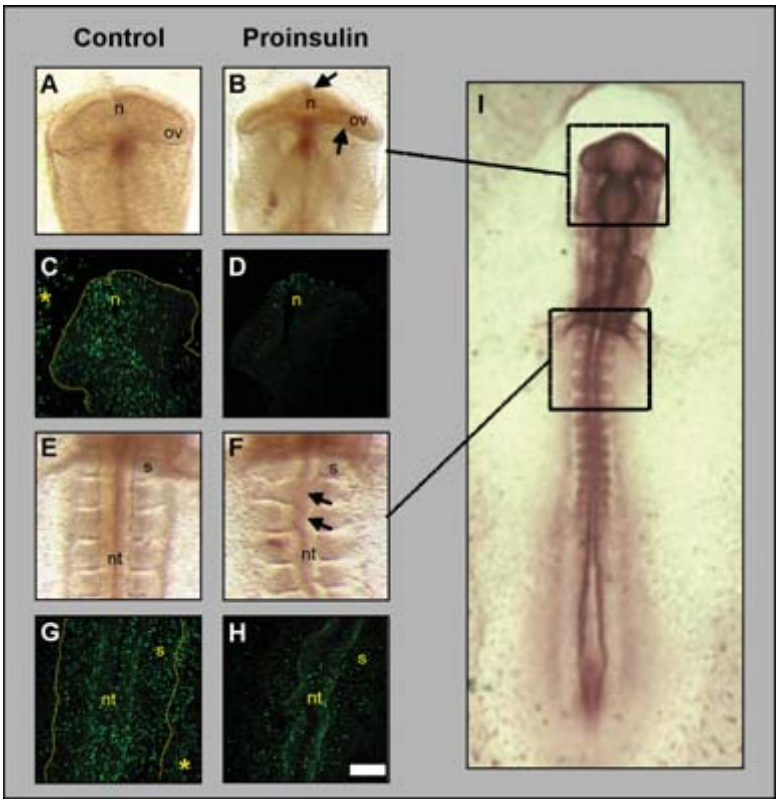

FIGURE 4. ***In ovo treatment with exogenous proinsulin reduces cell death and causes malformations***. *Embryos were treated in ovo with vehicle (A, C, E and G) or proinsulin (B, D, F and H) and after 8 h, the morphology of the embryos was evaluated by counterstaining with neutral red (A, B, E and F). Apoptotic cells were visualized by TUNEL staining of whole embryos (C, D, G and H), and serial images (z-axis) were captured with a confocal microscope every 10 μm and compiled. For orientation, the different regions shown are boxed in a photograph of the whole embryo (I). The upper box corresponds to the prosencephalon (A-D) and the lower box to neural tube and rostral somites (E-H). The main morphological features are labeled: n, anterior neuropore; ov, optic vesicle; nt, neural tube; s, somite. Arrows in (B) and (F) indicate the main morphological abnormalities [reproduced with permission from (22); © 2003 European Molecular Biology Association]. Calibration bar, 120 μm (A-H) and 200 μm (I).*

with caspase inhibitors decreases PCD and interestingly, increases the number of RGCs (Figure 7).

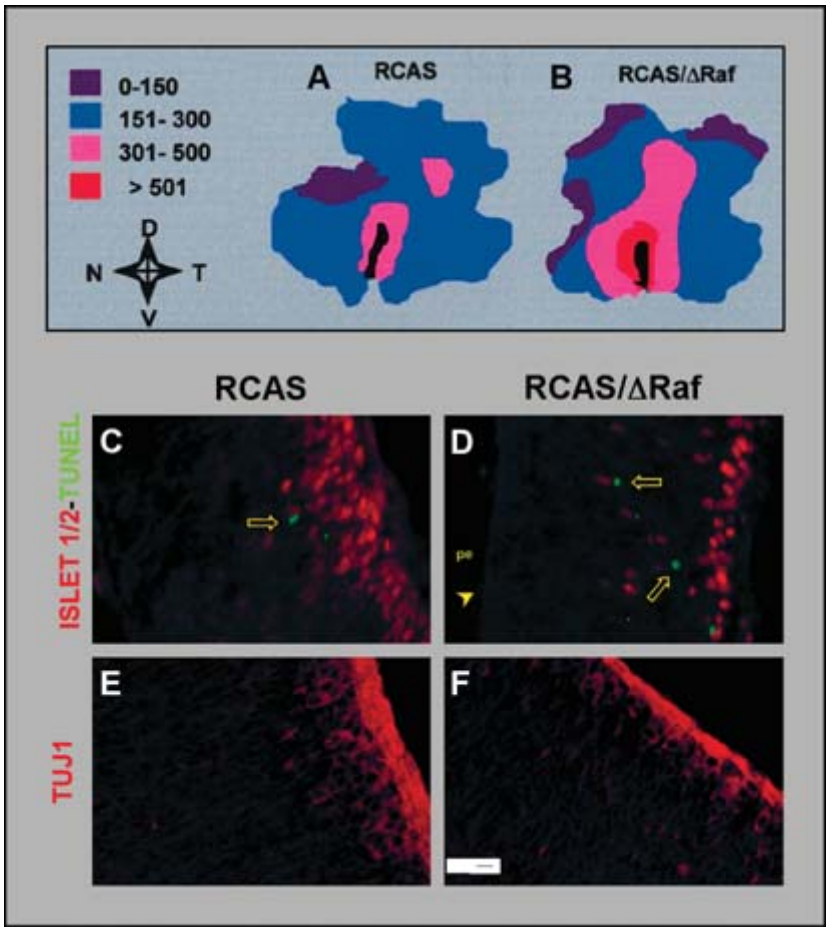

FIGURE 5. ***Interference with c-Raf causes cell death and disturbs RGC morphogenesis.*** *E4.5 chick embryos were injected intravitreally with a retrovirus carrying the indicated viral constructs (RCAS, empty vector; RCAS/ΔRaf, c-Raf dominant negative construct) and their retinas whole-mount TUNEL stained 48 h later (A, B) or immunostained as cryosections 72 h later (C-F). The density of dead cells was represented as isothanas (A, B), the pseudocolor scale indicating TUNEL-stained apoptotic bodies per square millimeter. The orientation of the retinas is indicated: N, nasal; T, temporal; D, dorsal; and V, ventral. Retinal sections were double-stained for TUNEL (green, arrows, in C, D) and the neuronal marker Islet 1/2 (red in C, D). Adjacent sections were stained for the neuronal marker TUJ1, which stains the optic fiber layer (red in E, F). The side of the pigmented epithelium (pe) is indicated (arrowhead) [reproduced with permission from (26); © 2000 Society for Neuroscience]. Calibration bar, 20 μm (C-F).*

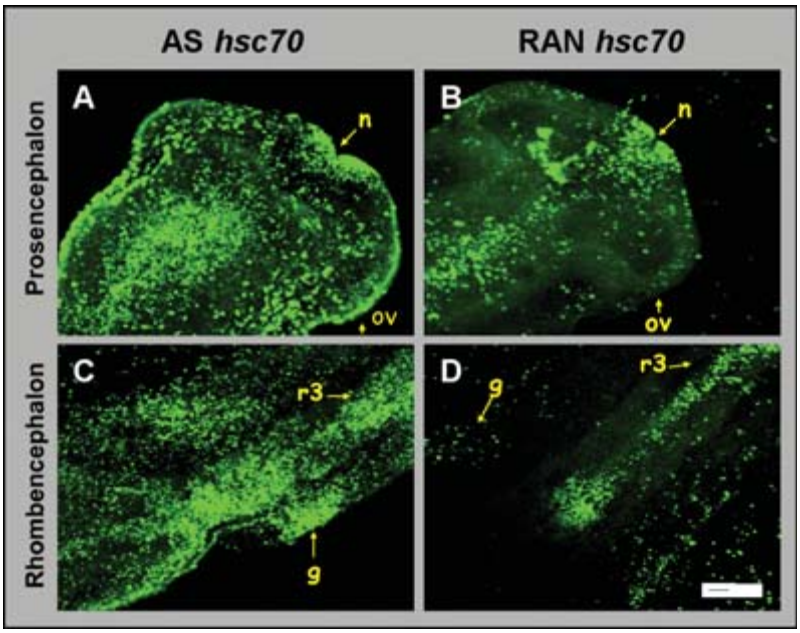

FIGURE 6. ***Interference with hsc70 causes cell death in neurulating chick embryos***. *Apoptotic cells visualized by TUNEL staining of whole HH10 embryos cultured for 8 h in the presence of either antisense ODNs to hsc70 (AS hsc70; A and C) or random control ODNs (RAN hsc70; B and D). Serial images (z-axis) were captured every 10 μm with a 10× objective on a confocal microscope and compiled: prosencephalon (A, B), rhombencephalon (C, D). The main morphological features are labeled: g, presumptive otic vesicle anlage; n, anterior neuropore; ov, optic vesicle; r3, rhombomere 3 [reproduced with permission from (23); © 2002 Federation of European Neuroscience Societies]. Calibration bar, 200 μm.*

FUTURE PERSPECTIVES

Perturbing RGC generation is a common result of our *in ovo* manipulations. Interference with prosurvival signals, such as proinsulina/insulin or c-Raf, reduces the number of RGCs by 50% (see Figures 3 and 5) (20, 26), whereas blocking cell death doubles the number of RGC (see Figure 7) (24). This susceptibility of RGCs to death, together with a detailed analysis of the available knock-out mouse models should provide valuable tools to determine the functional role of early neural cell death (9, 17).

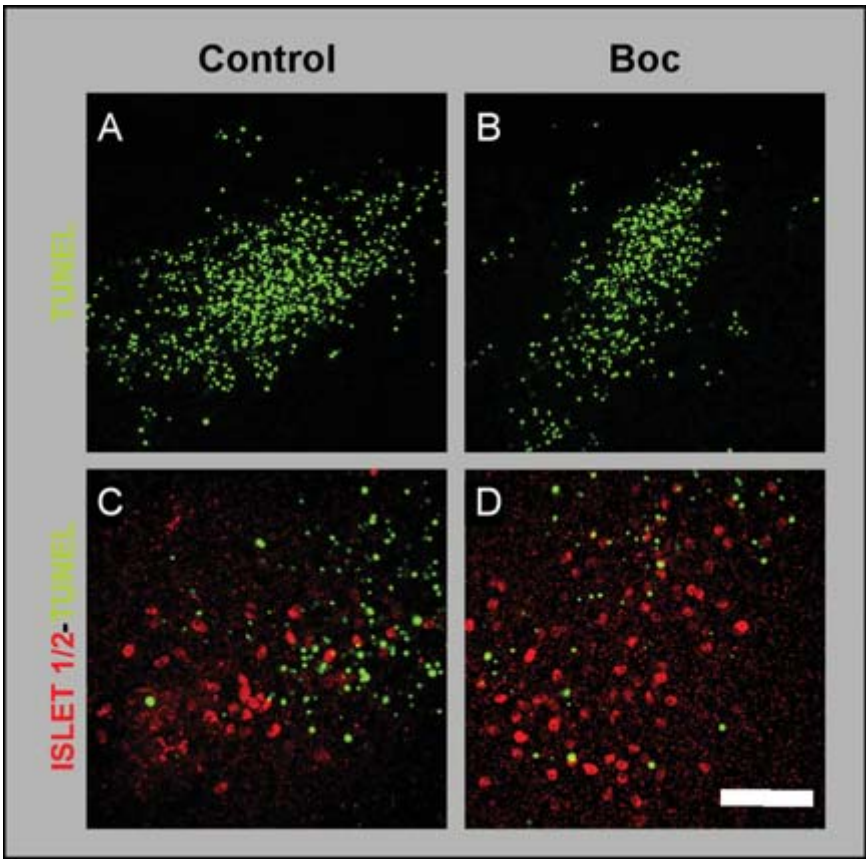

FIGURE 7.- ***Effect of caspase inhibition on the generation of RGCs.*** *HH17 chick embryos were treated in ovo for 6 h with either vehicle (Control; A and C) or with the broad-range caspase inhibitor Boc-D-fmk (Boc; B and D). Following treatment, whole-mount retinas were stained for TUNEL (green in A-D) to visualize apoptotic cells, and Islet 1/2 (red in C, D) to identify RGCs. (C) and (D) correspond to higher magnification fields, where the increase in the number of Islet 1/2 positive cells can be observed [reproduced with permission from (24); © 2003 Federation of European Neuroscience Societies]. Calibration bar, 200 μm (A, B) and 50 μm (C, D).*

In addition, since deregulated cell death seems to be associated with the progress of neurodegenerative disorders, new findings on the regulation of physiological cell death during neural development may be extrapolated to pathological conditions (31-34). Indeed, a

preliminary exploration of the neuroprotective role of proinsulin has rendered promising results in a mouse model of retinitis pigmentosa. Neurodegenerative diseases have a dramatic impact not only the patients and their families but also, on society as a whole. Thus, there is a need for intense basic and oriented research to provide new therapeutic approaches to prevent neurodegeneration.

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