Development 127, 4561-4572 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV1595

Cell interactions within nascent neural crest cell populations transiently promote death of neurogenic precursors

Thomas M. Maynard, Yoshio Wakamatsu and James A. Weston*

Institute of Neuroscience, University of Oregon, Eugene, OR 97403-1254, USA
*Author for correspondence (e-mail: weston@uoneuro.uoregon.edu)

Accepted 18 August; published on WWW 9 October 2000

SUMMARY

We have previously shown that cultured trunk neural crest cell populations irreversibly lose neurogenic ability when dispersal is prevented or delayed, while the ability to produce other crest derivatives is retained (Vogel, K. S. and Weston, J. A. (1988) *Neuron* 1, 569-577). Here, we show that when crest cells are prevented from dispersing, cell death is increased and neurogenesis is decreased in the population, as a result of high cell density. Control experiments to characterize the effects of high cell density on environmental conditions in culture suggest that reduced neurogenesis is the result of cell-cell interactions and not changes (conditioning or depletion) of the culture medium. Additionally, we show that the caspase inhibitor zVAD-fmk, which blocks developmentally regulated cell death, rescues the neurogenic ability of high density cultures, without any apparent effect on normal, low-density cultures. We conclude, therefore, that increased cell interaction at high cell densities results in the selective death of neurogenic precursors in the nascent crest population. Furthermore, we show that neurogenic cells in cultured crest cell populations that have dispersed immediately are not susceptible to contact-mediated death, even if they are subsequently cultured at high cell density. Since most early migrating avian crest cells express Notch1, and a subset expresses Delta1 (Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000) *Development* 127, 2811-2821), we tested the possibility that the effects of cell contact were mediated by components of a Notch signaling pathway. We found that neurogenic precursors are eliminated when crest cells are co-cultured with exogenous Delta1-expressing cells immediately after they segregate from the neural tube, although not after they have previously dispersed. We conclude that early and prolonged cell interactions, mediated at least in part by Notch signaling, can regulate the survival of neurogenic cells within the nascent crest population. We suggest that a transient episode of cell contact-mediated death of neurogenic cells may serve to eliminate fate-restricted neurogenic cells that fail to disperse promptly in vivo.

Key words: Neural crest, Cell fate, Cell death, Neurogenesis, Delta-Notch, Quail

INTRODUCTION

In the trunk of the avian embryo, neural crest cells segregate from the neuroepithelium into a migration staging area (MSA; Weston, 1991) adjacent to the dorsal neural tube. They then disperse along spatially and temporally distinct pathways to form a variety of neuronal and non-neuronal cell types (Le Douarin and Kalcheim, 1999). Because they produce a diverse array of cell types, each appropriate for a particular embryonic location, trunk neural crest cells have served as a useful paradigm for learning how environmental cues affect lineage diversification during embryonic development.

Although a variety of cell culture studies reveal that some nascent neural crest cells may be multipotent (Anderson, 1993; Bronner-Fraser, 1993; Sieber-Blum, 1989), it is now clear that soon after it emerges from the neural tube the majority of the crest cell population is composed of bi-potent or fate-restricted cells (Baroffio et al., 1988; Erickson and Goins, 1995; Henion and Weston, 1997; Sieber-Blum, 1999). These results suggest that the premigratory neural crest population is a heterogeneous mixture of several developmentally distinct subpopulations, which, in vivo, give rise to the different cell phenotypes in appropriate embryonic locations.

Normally, avian and murine trunk neural crest cells that disperse early on a ventromedial migration pathway give rise to neurons and glia in peripheral ganglia, whereas cells that disperse late on the lateral migration pathway produce melanocytes and have very limited neurogenic ability (see Wakamatsu et al., 1998). The allocation of cells to each embryonic location presumably involves a number of mechanisms. For example, migration of fate-restricted precursors can be directed to specific embryonic locations by localized tropic cues (Wehrle-Haller and Weston, 1997). Alternatively, ectopically localized cells can be selectively 'edited' by cell death (Raff et al., 1993; Wakamatsu et al., 1998). Finally, lineage choices among neighboring crest-derived precursors can be regulated by asymmetrical cell divisions and cell interactions (Wakamatsu et al., 2000). It is not clear, however, what mechanism(s) assure that early migrating cell populations are primarily neurogenic, whereas

populations that undergo delayed migration generally lack neurogenic ability.

Previous work has demonstrated that cultured trunk neural crest cell populations progressively and irreversibly lose the ability to produce neurons when their dispersal is prevented or delayed (Vogel and Weston, 1988). In view of the apparent developmental heterogeneity of premigratory crest populations, we previously suggested that loss of neurogenic ability by the crest cell population could be due to the elimination of a subpopulation of neurogenic precursors when the cells failed to disperse. It was not known, however, whether such elimination was the result of selective death of the neurogenic subpopulation, or if the neurogenic cells assumed a non-neuronal fate. As is often the case, moreover, the relevance of this in vitro phenomenon to developmental events in vivo was unclear.

In principle, cell dispersal can change many aspects of the environment encountered by crest cells, including their exposure to diffusible factors, their contact with other cells or their association with extracellular matrix. Each of these environmental cues has been implicated in the regulation of differentiation of numerous cell types (Calof, 1995), and therefore might be involved in the loss of neurogenic potential in the population. Here, we have confirmed the identity of the affected cells and have examined some environmental consequences of preventing cell dispersal. Our results indicate (1) that the loss of neurogenic ability by crest cell populations that fail to disperse promptly is due to the death of neurogenic precursors, (2) that death of neurogenic precursors is caused by increased cell interactions, and (3) that such cell interactions can initiate Notch-Delta signaling, which we suggest is transiently able to induce death of fate-restricted neurogenic cells in the nascent crest population.

MATERIALS AND METHODS

Culture medium

Quail crest cell culture medium is based on Ham's F12 medium (Gibco), supplemented with 15% fetal bovine serum (Hyclone, Logan, Utah), 4% E10 chicken embryo extract, penicillin/streptomycin and 10 mM Hepes (Sigma), pH 7.5. Batches of serum and embryo extract were tested for ability to support the survival and differentiation of both neurons and melanocytes, and the same batches of serum and embryo extract were used for the majority of the experiments reported here.

The S2 cell lines were grown in Schneider's medium (Gibco), supplemented with 10% fetal bovine serum, penicillin/streptomycin and 10 mM Hepes. The L cell lines were grown in Dulbecco's minimum essential medium with high glucose, also supplemented with 10% fetal bovine serum, penicillin/streptomycin and Hepes.

Neural crest cell cultures

Undispersed neural crest cell populations (neural crest clusters) were isolated as previously described (Glimelius and Weston, 1981; Loring et al., 1981). Briefly, embryonic neural tubes were isolated from the level of the last 6-8 somites from 17-22 somite quail embryos (equivalent to stage 13-14 chick embryos, Hamburger and Hamilton, 1951). These neural tubes were incubated with pancreatin (Gibco) in Hanks' balanced salt solution (HBSS), triturated to remove attached mesodermal tissues and cultured on non-adhesive agar coated dishes. At intervals after initiation of primary culture, clusters of neural crest cells were dissected from the primary neural tube cultures with tungsten needles, washed and dissociated by a mild treatment with diluted trypsin-EDTA in HBSS. The lowest concentration of trypsin that would dissociate crest cell populations without shearing was used; this concentration depended on the batch of enzyme, but was generally approximately 0.005%. Cells were then thoroughly washed and resuspended in complete culture medium before counting in a hemacytometer. To enhance cell recovery, the dissociation and wash steps included 10% normal culture medium added to the Ca^{2+}- and Mg^{2+}-free Hanks' buffer (CMF-HBSS).

Dispersed crest cells (neural tube outgrowths) were prepared by placing explanted neural tubes directly onto tissue culture dishes (Corning) in 10 mm Sylgard (Dow Corning) wells filled with medium. After initial culture, the neural tube explants were removed from the substratum with tungsten needles and the debris washed away with warm HBSS. The adherent outgrowth population was then harvested by dissociating them as described above.

To determine the neurogenic ability of crest cell populations, crest cells from populations derived from both clusters and neural tube outgrowths were cultured in 5 mm Sylgard wells placed on tissue culture plastic dishes in approximately 50 μl of medium (see Fig. 1A). Approximately half the medium in each well was replaced each day, and the cells were grown for a total of 5 days, after which they were fixed and immunostained as described below.

To prepare cultures at varying cell densities, four 5 mm Sylgard wells were placed in a single 35 mm culture dish, and different numbers of dissociated crest cells were added to each well in approximately 30 μl of medium (see Fig. 2A). These cells were incubated for 30 minutes to allow the cells to adhere to the dish, then 3 ml of medium was gently added to the dish to cover all four wells. In each experiment, aliquots of the same cell suspension were introduced into corresponding wells of duplicate culture dishes. One dish was cultured for 4 days and neurogenesis in each well was assessed. The cells in the wells of the other dish were fixed after 6 hours for TUNEL analysis. Because plating efficiency of any primary culture is variable, the 6 hour wells were also used to determine directly the actual cell density of the sister wells in the dish that had been cultured for 4 days.

To assess the role of cell density, independent of cell number or association with matrix proteins, cultures were prepared by suspending 80,000 dissociated crest cells in a collagen gel (Altshuler and Cepko, 1992). Cells were initially suspended in quail medium containing 250 mM Hepes buffer (pH 7.5), and this suspension was mixed 2:1 with collagen (Rat tail type I, Upstate Biotechnology, Lake Placid, NY) in a microcentrifuge tube. Cells were suspended in either 200 μl or 40 μl of collagen, or cultured as pellets overlaid with 200 μl of collagen. After a 30-minute incubation, the solidified gel was overlaid with normal medium for culture. A 'balancer' plug of 160 μl of gel was added to the smaller (40 μl) gel suspension at this time, so that the cultures all contained the same amount of collagen. After an initial culture period in the collagen gel, cells were removed from the collagen matrix by adding collagenase D (Boehringer-Mannheim Biochemicals) to the gel and medium to a final concentration of 0.1%. After a 15 minutes incubation at 37°C, the cells were thoroughly washed in normal quail medium (see Fig. 3A).

To assess the effect of retinoic acid, which selectively promotes neurogenesis in crest populations (Henion and Weston, 1994), high-density 'pellet' cultures which were prepared as above by adding 80,000 dissociated crest cells in 500 μl of culture medium to a siliconized microcentrifuge tube (Biorad), then gently pelleting the cells by centrifugation. A 10 mM stock solution of all *trans* retinoic acid (RA; Sigma) in DMSO was then added to a final concentration of 10 nM. Control cultures received an equivalent dilution of DMSO. Dispersed cell controls (prepared as above: 10,000 cells/5 mm well) were also prepared in culture medium to which RA in DMSO or DMSO alone was added. After secondary culture, the cell pellets were again dissociated by brief treatment with dilute trypsin-EDTA, then washed and resuspended in complete culture medium. A portion of these cells was then plated to assess neurogenic ability, while the

remaining cells were fixed and processed in suspension to measure the number of TUNEL-positive cells. At the same time, the dispersed cell controls were thoroughly washed with warm HBSS and untreated culture medium (see Fig. 4A).

To assess the effect of apoptosis on neurogenesis, some pellet cultures were treated with a membrane-permeant modified tripeptide (zVAD-fmk; Bachem), that competitively and specifically inhibits the proteolytic function of cell-death-specific caspase proteins (Jacobsen et al., 1996; Weil et al., 1996). zVAD-fmk was added from a 100 mM stock in DMSO to a final concentration of 50 μM.

To compare the effect of high-density culture on neurogenesis by outgrowth- and cluster-derived crest cells, cells from these two sources were dissociated and pelleted in microcentrifuge tubes as described, maintained for 6 or 18 hours in the centrifuge tubes before replating in 5 mm Sylgard wells at a cell density known to be permissive for neurogenesis (10,000 cells/well) and culturing for 4 days (see Fig. 5A).

Exposure of cultured crest cells to Delta1-expressing cells

To activate the notch signaling pathway in crest cells, we co-cultured them with two cell lines that were made to express a quail Delta1 homolog constitutively. The sequences of the first and last 20 base pairs of chicken Delta1 homolog (Henrique et al., 1995; Myat et al., 1996) were used to generate primers for PCR of a quail cDNA library prepared from RNA extracted from 6 day quail embryo dorsal root ganglia. PCR products encompassing the full coding sequence of the quail Delta1 protein were then produced and cloned into pBluescript. Sequencing of quail Delta1 revealed that it is virtually identical to the chicken sequence (97.5% homology; T. M. Maynard, PhD Thesis, University of Oregon, 1999).

Both adhesive (L^{tk-}; ATCC) and non-adhesive (S2; Schneider, 1972) cells were transfected. These parental cell lines possess two important characteristics: neither endogenously expresses any Delta-family ligand, and both have been successfully used to express functional Notch and Delta family proteins by others (Fehon et al., 1990; Lindsell et al., 1995). The adhesive Delta1-expressing cell line was produced by inserting the Delta1 coding sequence into the pcDNA 3.1 expression vector (Invitrogen), transfecting L^{tk-} cells, and selecting clonal cell lines that were resistant to G418 (Gibco). Quail Delta1 expression by these cells was assessed by live immunostaining with anti-Delta antiserum (Wakamatsu et al., 2000; data not shown). Before use as feeder layers in crest cell cultures, L-cells were treated for 2 hours with 10 μg/ml mitomycin C to block further cell division, washed thoroughly, then dissociated by washing with CMF-HBSS. Cells were then resuspended in quail medium, and counted on a hemocytometer.

A non-adhesive Delta-expressing cell line was produced by replacing the CMV promoter of the Delta-pcDNA vector with the *Drosophila* Actin5C promoter from pP-Ac (Krasnow et al., 1989), and co-transfecting a *Drosophila* cell line, S2 (Schneider, 1972) with this vector and a second vector containing the Actin 5C promoter with the pcDNA 3.1 neo gene. Selecting these cells with G418 produced a population of cells that constitutively expressed Delta, as assessed by live immunostaining with anti-Delta antiserum (data not shown). For co-cultures, batches of these cells were harvested, washed thoroughly in CMF-HBSS, resuspended in quail medium, and counted on a hemocytometer.

To test the effects of Notch activation on crest cell death and differentiation, cluster-derived crest cells or crest cells from neural tube outgrowths were co-cultured with Delta1-expressing cells. Short-term cultures of crest cells were harvested from 20-24 hour crest cell clusters and dispersed as described above and then assayed for the proportion of dying (TUNEL-positive) cells as follows. After dispersal, approximately 4000 crest cells were co-cultured with 8000 adhesive Delta1-expressing or control L cell lines. Because the L cells are larger than the avian crest cells, the two cell types could be easily distinguished and the crest cells were primarily in contact with the L cells and not with each other. The identity of crest cells in some cultures was confirmed by immunostaining with QCPN antibody (Developmental Studies Hybridoma Bank, University of Iowa). After a 6 hour co-culture with quail Delta1-expressing or control L cell lines, the proportion of TUNEL-positive crest cells was assessed.

Alternatively, to test the effect of longer interaction between crest cells and exogenous Delta1-expressing cells, neural tube segments were cultured in 5 mm Sylgard wells (two segments/well). At defined intervals, 180,000 Delta1-expressing or control (parental) S2 cells were added to the cultures (Fig. 6A). After each 24-hour interval, culture wells were gently rinsed with 10% complete medium in HBSS, then thoroughly washed with fresh complete media. This procedure removed the majority of the S2 cells, while leaving the avian crest cells attached to the culture substratum.

Some co-cultures were treated with zVAD-fmk (Bachem; see above), added from a 100 mM stock in DMSO to a final concentration of 100 μM. In the S2 co-cultures, the caspase inhibitor was added along with the S2 cells, and both the drug and the overlay cells were washed away after 24 hours.

Immunocytochemistry and TUNEL

To identify neuronal cells in cultured crest cell populations, paraformaldehyde-fixed cells were exposed to the pan-neuronal anti-Hu monoclonal antibody, 16A11 (Marusich et al., 1994), and antibody binding was detected with a FITC-conjugated anti-mouse IgG secondary antibody (Southern Biotechnology). After thorough washing, the cells were counterstained with DAPI and mounted in glycerol for photography. For in situ immunostaining, we used an alkaline-phosphatase-labeled secondary antibody (Southern Biotechnology) followed by treatment with NBT/BCIP to detect Hu-positive cells (see Wakamatsu and Weston, 1997).

The in situ TUNEL technique (Gavrieli et al., 1992; Gold et al., 1993) was adapted to identify apoptotic cells in culture. Paraformaldehyde-fixed cells, either in suspension or in culture wells, were labeled by incubating for 60 minutes at 37°C with 125 U/ml terminal transferase and 5 pM Fluorescein-dUTP (Boehringer) in a TUNEL reaction buffer (25 mM Tris, 200 mM sodium cacodylate, 1mM cobalt chloride, 25 mg/ml BSA, 0.2% Tween-20, pH 6.6). After washing in Tris-buffered saline (TBS), samples were counterstained with DAPI and observed with a Zeiss epi-fluorescence microscope. Suspended cells were counted on a hemocytometer, while adherent cells were mounted with glycerol and a representative field was photographed in each fluorescent channel for analysis.

Cell counts and statistics

Representative fields of each cell culture were chosen and each channel (DAPI and FITC for TUNEL labeled cells; DAPI, FITC and bright-field views for long term cultures) was photographed onto 35 mm slide film for cell counts. Each set of slides was scanned into Photoshop (Adobe), then assembled into a composite image overlaid with a counting grid. A bacterial colony counter was used to count the number of positive cells observed in each channel. An area of at least 50 μm^2 was analyzed for every sample, although additional area was examined for low density samples (<200 cells/50 μm^2).

Statistical analysis of cell ratios was performed by non-parametric methods (Fisher and Van Belle, 1993) using StatView software (SAS Institute). Comparisons of two sample populations were made using the Mann-Whitney U-test. Correlation analyses used the Spearman rank-order test, with values corrected for ties where necessary. Error bars in all figures represent standard error (s.e.m.).

RESULTS

Cell death is increased when crest cell dispersal is prevented

Crest cell populations progressively and irreversibly lose the

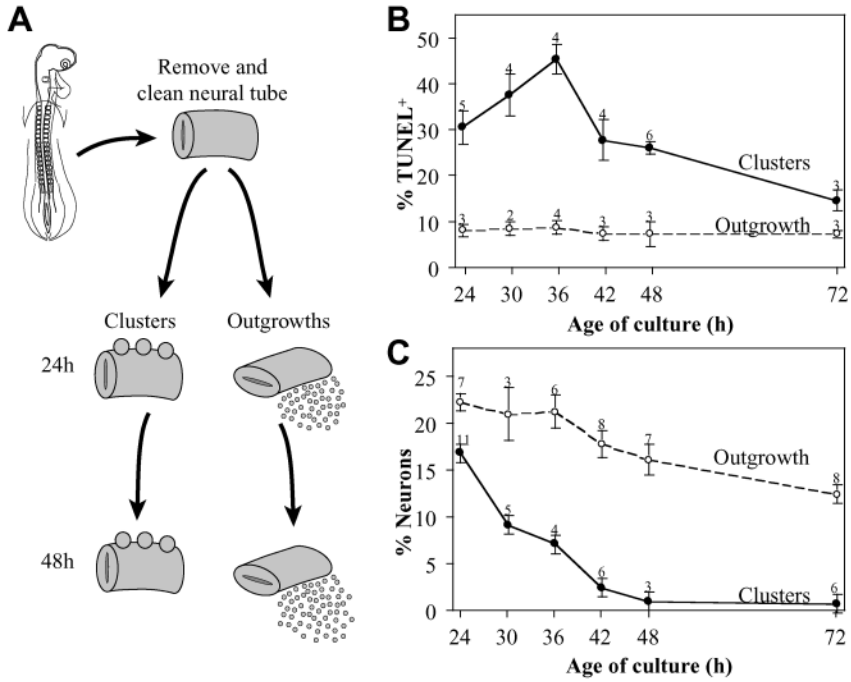

Fig. 1. Loss of neurogenic potential in undispersed crest cell populations correlates with elevated levels of cell death. (A) Schematic of experimental procedure, showing the two crest cell populations that were used as the source of cells. (B) The level of cell death is higher in undispersed (cluster-derived) crest cell populations during the first 18 hours of culture than in populations of cells that disperse immediately (neural tube outgrowth). (C) The level of neurogenesis (Hu immunoreactivity) declines rapidly in populations derived from crest cell clusters, whereas outgrowth-derived crest cell populations lose neurogenetic ability slowly. Error bars denote standard error; the number of experiments, *n*, is denoted next to error bar for each data point.

ability to produce neurons when their dispersal is delayed in vitro (Vogel and Weston, 1988). To determine quantitatively whether delayed dispersal of crest cell populations also affects cell survival, we assessed the level of cell death in two cultured neural crest populations – cells that were not allowed to disperse (neural crest clusters), and a control population that had dispersed immediately (neural tube outgrowths; see Fig. 1A). We dissociated crest cells from both of these populations at several different times during primary culture and measured the level of cell death with a modified TUNEL assay (Gavrieli et al., 1992). Aliquots of these cell suspensions were then grown in standardized secondary cultures, differing only in the source of cells, to assess their neurogenic ability. A neuron-specific monoclonal antibody to the Hu family of RNA-binding proteins (16A11, Marusich et al., 1994; Wakamatsu and Weston, 1997) was used to identify both differentiating and mature neurons in these cultures. The proportion of TUNEL-positive cells in each population was then compared with the proportion of neurons that were produced from sister cultures of the same population.

The proportion of TUNEL-positive cells in populations obtained from crest cell clusters between 24 and 36 hours of culture was much greater than in cell populations obtained from older clusters (Fig. 1B). TUNEL was highest at the time when the decline in neurogenic potential was the greatest (Fig. 1C), and was lowest after 36 hours, when the neurogenic potential of the population had largely disappeared. In contrast, cultures of dispersed crest cells harvested from outgrowths showed neither the dramatic decline in the proportion of neurons, nor the high levels of cell death observed in the populations that had been prevented from dispersing. These data show that the loss of neurogenic ability by the neural crest population is coincident with a transient period of cell death. To elucidate the mechanism responsible for this decrease, we addressed two broad issues. First, we examined what aspect of the altered environment caused by delayed dispersal leads to the loss of neurogenic potential and concomitant cell death. Second, we investigated the identity of the dying cells.

Cell death in cultured crest populations increases at high cell density

Among other things, delayed dispersal prolongs the time during which crest-derived cells remain at high cell population densities. High cell densities, in turn, can affect a number of environmental parameters including the ability of cells to interact with each other or their matrix substrata, as well as depleting nutrient components or conditioning the local environment with diffusible factors. To distinguish these contingent effects of the cells' environment, we performed a number of experiments to alter cell density while maintaining other conditions constant. To do so, crest cell populations that had not previously been allowed to disperse (24 hour clusters) were gently dissociated and added to culture wells at several concentrations. After the cells had adhered to the substratum within each well, a large volume (3 ml) of culture medium was added to the dish (Fig. 2A). Half of this medium was changed each day and mixed thoroughly to minimize local conditioning. We also assessed the extent to which medium components might diffuse from the culture wells by introducing bromophenol blue into test culture wells submerged in medium. Because the dye rapidly diffused from wells into the bulk medium even without agitation (data not shown), it appears unlikely that observed effects of cell density on cell fate could be attributed to addition or depletion of diffusible components in the nutrient environment surrounding the cells.

After 6 hours of culture under the conditions described above, a direct correlation between plating density and the level of cell death was observed (Fig. 2B, $P<0.05$ by Spearman rank-order correlation test). At high measured cell densities (>4000 cells/mm^2), resulting from plating cells at concentrations greater than approx. 75,000 cells/well, over 20% of cells were labeled by TUNEL, whereas at low cell densities (<500

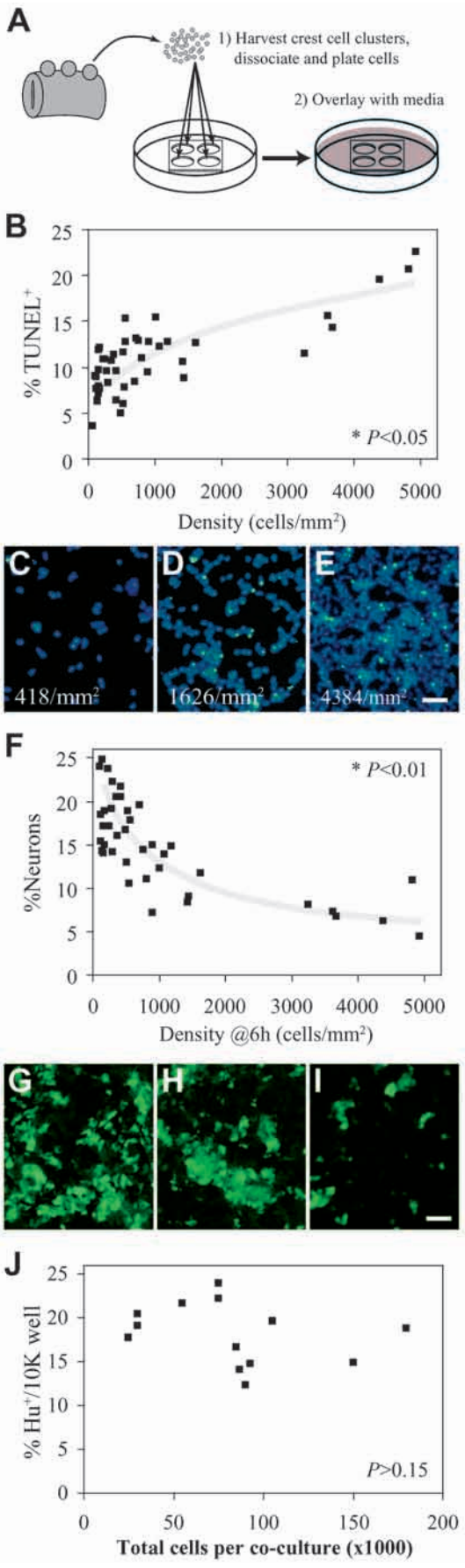

Fig. 2. Neurogenesis and cell death are correlated with plating density. (A) Schematic of experimental procedure. (B) Level of cell death (TUNEL-positive cells) observed after 6 hours of culture correlated with the measured density for that sample. (C-E) Representative TUNEL for three cell densities, indicated at bottom left. Green indicates TUNEL positive cells, blue indicates cell nuclei (DAPI staining). (F) Neurogenesis (Hu immunoreactivity) after 4 days correlated with the initial cell density measured at 6 hours in sister cultures. The shaded line indicates the data trend; however, the significant correlation between cell density and either TUNEL (*P<0.05) or neurogenesis (*P<0.01) was obtained using the Spearman rank-order correlation test. (G-I) Representative 16A11 Hu antibody staining of cultures at the same three plating densities shown in sister cultures (C-E). (J) Neurogenesis measured after 4 days of culture in 13 wells each with average effective plating density of 468±91 (s.e.m.) cells/well (achieved by adding 10,000 dissociated cells to each well) co-cultured with varying numbers of cells in the other wells of the same dish. Scale bar: 50 μm.

cells/mm^2), resulting from plating cells at concentrations less than approx. 15,000 cells/well, the proportion of TUNEL-positive cells was greatly reduced. At low cell densities, moreover, dying cells generally appeared only among clusters of cells. Sister cultures at equivalent cell densities, which had been allowed to develop for 4 days, showed a negative correlation between the level of neurogenesis and cell density (Fig. 2F, P<0.01). Since cell death and neurogenesis are both correlated with the initial plating density, we examined whether they were also correlated to each other. As expected, the level of neurogenesis observed at 4 days is strongly negatively correlated with the level of cell death in the 6 hour sister cultures (r=–0.45; P<0.05).

Next, we examined the level of neurogenesis in wells containing cells plated at the *same* density (10,000 cells/well, which in this case, yielded a plating density of 276±50 cells/mm^2), while the total number of cells sharing the culture medium in the same dish varied. In such cultures, no correlation was observed between the level of neurogenesis and the total number of cells that shared the same medium in the co-culture wells (Fig. 2J; P>0.15). Similarly, no correlation was observed with the level of cell death observed at 6 hours (P>0.30; data not shown). Taken together, these results demonstrate that high cell density itself, rather than alterations in the diffusible environment, promotes cell death and the concomitant loss of neurogenic ability in the cultured crest cell populations.

Cell contact in high-density cultures mediates cell death

Although the previous experiments suggest that cell death and loss of neurogenic ability are probably not due to differences in diffusible factors in the environment, two other possibilities – interaction between cells and their substratum, and interaction between the cells themselves – remain to explain the results. Because the cells continue to proliferate, aggregate, differentiate and die throughout the culture period, neither the degree of contact between cells and the substratum, nor the amount of contact between cells themselves can be readily controlled in mass cultures, and consequently these conditions are likely to change during the culture period. To address this issue, we gently dissociated previously undispersed (cluster-derived) crest cells and suspended them

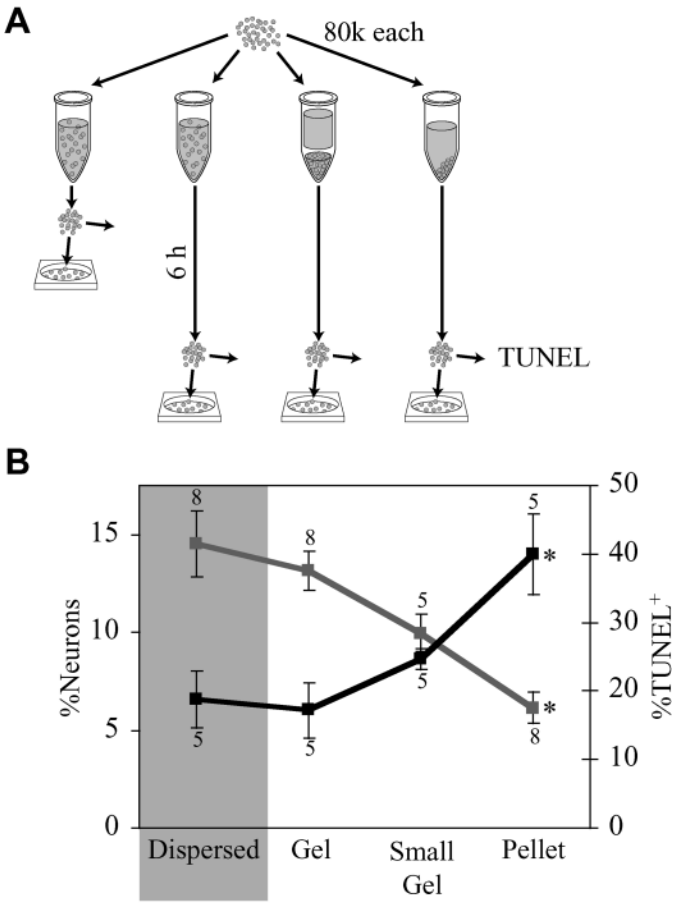

Fig. 3. Cell density is correlated with neurogenesis and cell death. (A) Schematic of experimental procedures. (B) Level of neurogenesis (gray line, Hu immunoreactivity) and level of cell death (black line, TUNEL positive cells) are both correlated with the density of gel culture. Number of cultures for each condition is indicated next to error bars. Note that immediately dispersed cells (shaded box) are not significantly different from low-density gel cultures (* P<0.01 by Spearman rank-order correlation test).

for a 6 hour period at various densities in a collagen matrix. Each population was then sampled to determine the proportion of TUNEL-positive cells, replated at a standard density, and incubated for several days to assess its neurogenic potential (Fig. 3A). Cells suspended in collagen gel are prevented from aggregating at high density, but retain access to matrix components, growth factors and nutrient medium (Altshuler and Cepko, 1992; Austin et al., 1995). To control for any nonspecific effects of these manipulations, each of these gel cultures was compared with cells suspended at low density in identical gels, and then immediately removed from the collagen and replated on normal culture substrata. Thus, the experimental design normalized other culture conditions so that the effect of increased cell contact-mediated interactions on cell fate could be assessed.

In these experiments, no significant differences in either TUNEL or neuronal staining were observed between crest cells suspended for 6 hours in the lowest density gel and cultures that were initially suspended at the same density in a collagen gel and then dispersed immediately (Fig. 3B; TUNEL, $P>0.3$; Hu, $P>0.5$). This result indicates that suspension in the gel did not itself alter these parameters. In contrast, there was a highly significant difference in both measures among the cells suspended in collagen gel at higher cell densities. Specifically, a significant correlation was observed between the density of the cells cultured in the collagen gel and the level of cell death ($P<0.01$), as well as between cell density and the level of neurogenesis ($P<0.01$). This suggests that even a transient (6 hour) period of elevated cell-cell interaction is sufficient to increase the level of cell death and to cause a concomitant decrease in the neurogenic potential of crest cell populations that have been prevented from dispersing. Furthermore, the inverse correlation observed between cell death and neurogenesis in the previous experiment is also evident in these transient high-density cultures.

Loss of neurogenic ability is caused by death of a neurogenic subpopulation of crest cells

Although the previous experiments indicate that delayed dispersal leads to increased cell death, the normal fate of the dying cells is unknown. More specifically, it is not known if the dying cells are primarily or exclusively the neurogenic precursor subpopulation. In the absence of a cell type-specific marker for such neurogenic precursors, it is not possible to confirm directly that death of such precursors is responsible for the loss of neurogenic ability by the crest cell population. However, it was possible to validate this inference indirectly. To do so, we examined two contingent issues: the level of cell death in the population when neurogenesis was promoted and, reciprocally, the level of neurogenesis in the population when cell death was prevented. We predicted that if the dying cells were neurogenic precursors, then conditions that selectively promoted their survival and differentiation would reduce the proportion of TUNEL-positive cells in the cultures. Conversely, we predicted that preventing cell death should increase neurogenesis in these populations.

To test the first prediction, we exploited the observation that retinoic acid (RA) at nanomolar concentrations selectively promotes neurogenesis in cultured crest cell populations (Henion and Weston, 1994). We harvested undispersed crest cells from neural crest clusters, and cultured them as high-density pellets for 6 hours in the presence or absence of RA (Fig. 4A). After 6 hours, both the level of cell death and the neurogenic ability of the population under standardized test conditions were assessed. As predicted, RA-treated pellet cultures contained significantly more neurons than control populations (Fig. 4B,J; $P<0.01$), as well as significantly fewer dying cells (Fig. 4C; $P<0.01$).

To test the second prediction, we treated pellets of crest cells with the anti-apoptotic drug zVAD-fmk. This drug, a tripeptide caspase inhibitor that has been shown to block cell death both in vitro and in vivo, causes no apparent effects on normal cell functions such as cell proliferation (Jacobsen et al., 1996). As predicted, however, pellet cultures treated with 50 μM zVAD-fmk contain fewer dying cells than control populations (Fig. 4C; $P<0.02$), as well as a significantly greater proportion of neurons (Fig. 4B,I; $P<0.001$).

It is important to note that, although both RA and zVAD-fmk treatments prevented the proportion of neurons in the cell pellets from declining, cultures of crest cell populations that had dispersed immediately produced the same proportion of neurons with or without the drug treatments (zVAD (Fig.

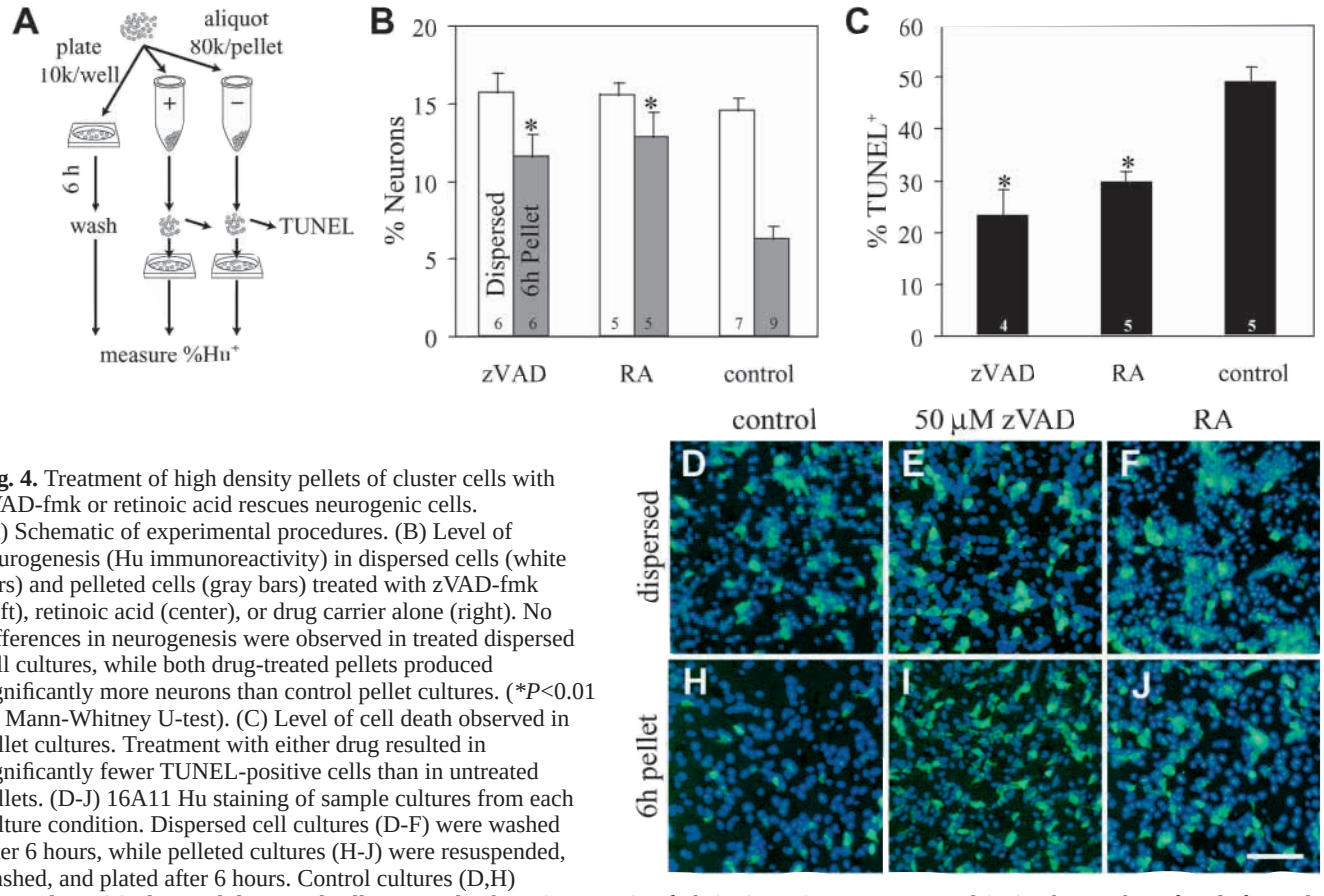

Fig. 4. Treatment of high density pellets of cluster cells with zVAD-fmk or retinoic acid rescues neurogenic cells. (A) Schematic of experimental procedures. (B) Level of neurogenesis (Hu immunoreactivity) in dispersed cells (white bars) and pelleted cells (gray bars) treated with zVAD-fmk (left), retinoic acid (center), or drug carrier alone (right). No differences in neurogenesis were observed in treated dispersed cell cultures, while both drug-treated pellets produced significantly more neurons than control pellet cultures. (*P<0.01 by Mann-Whitney U-test). (C) Level of cell death observed in pellet cultures. Treatment with either drug resulted in significantly fewer TUNEL-positive cells than in untreated pellets. (D-J) 16A11 Hu staining of sample cultures from each culture condition. Dispersed cell cultures (D-F) were washed after 6 hours, while pelleted cultures (H-J) were resuspended, washed, and plated after 6 hours. Control cultures (D,H) received DMSO alone, while treated cells received either 50 μM zVAD-fmk (E,I) or 10 nM retinoic acid (F,J). The number of trials for each condition are noted on each graph. (*P<0.05 by Mann-Whitney U-test.) Scale bar: 50 μm.

4B,E), P>0.30; RA (Fig. 4B,F), P>0.35). These results suggest that neither treatment induces neurogenesis, and confirms the inference that death of neurogenic precursors is responsible for the loss of neurogenic potential in the population.

Crest cells that disperse immediately are not susceptible to contact-mediated loss of neurogenic precursors

The foregoing experiments suggest that cell interactions lead to death of neurogenic precursors in the crest cell population. This result is paradoxical since overt neuronal differentiation by crest-derived cells in vivo usually takes place within the high cell density neighborhood of a developing ganglion (see Wakamatsu et al., 2000). Because the migrating crest-derived cells that undergo gangliogenesis represent an early migrating population, we reasoned that the initially migrating cells in vivo might correspond to the cell population that disperses immediately in vitro, which consequently would not be susceptible to cell-contact-mediated loss of neurogenic precursors. To test this prediction, we harvested crest cells that had dispersed immediately (outgrowths from 24 hour neural tube explants), and cultured them as high-density cell pellets for either 6 or 18 hours (Fig. 5A). We then replated the cells at a fixed density as previously described. Neurogenesis in these cultures was compared with that in cell populations prepared from cells that had not been allowed to disperse (24 hour crest cell clusters), and then were plated at the same density. The results unambiguously confirmed that neurogenesis was not decreased in cultures derived from crest cells that had been allowed to disperse immediately (Fig. 5B, outgrowths) even when they were subsequently cultured at high cell densities. In contrast, as predicted, neurogenesis in identically treated cultures derived from initially undispersed (cluster-derived) crest populations was reduced significantly (Fig. 5B, clusters).

Interaction with Delta1-expressing cells leads to the death of a neurogenic subpopulation of crest cells

We have recently reported that early migrating crest cells of avian embryos express *Notch1*, and that a subset of these cells expresses *Delta1*. In addition, we have shown that most Delta1-expressing crest cells in vitro have, or acquire, neuronal identity (Wakamatsu et al., 2000). We reasoned, therefore, that cell interactions leading to cell death could occur among early migrating crest cells, and that such effects might be mediated, both in vivo and in vitro, by Notch-Delta signaling. To test this idea, neural tube explants were overlaid with Delta1-expressing or control (parental) S2 cells for a 24 hour interval during one of the first 3 days of a 5-day culture period (Fig. 6A,B). To keep the treatment of each outgrowth population consistent, every outgrowth was washed daily and, at that time, each received fresh control or Delta1-transfected S2 cells. Because S2 cells adhere minimally to the culture substratum

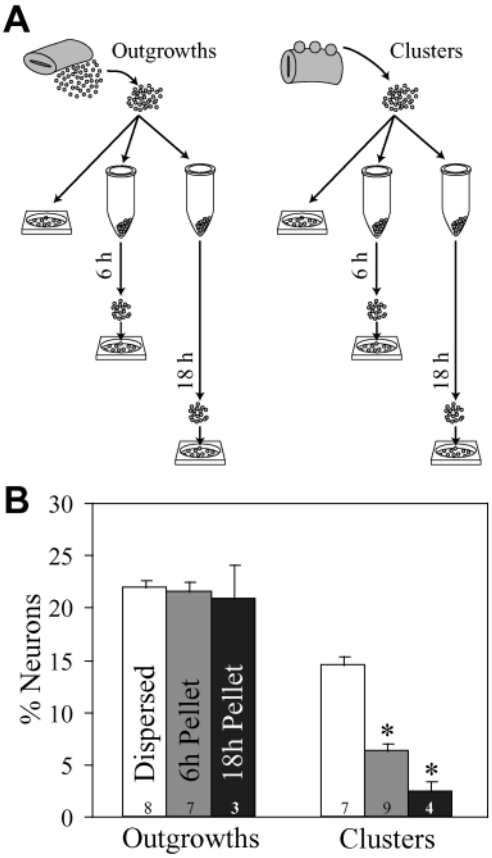

Fig. 5. Previously dispersed cells do not exhibit density dependence. (A) Schematic of experimental procedures. (B) Level of neurogenesis (Hu immunoreactivity) observed in cells harvested from outgrowth cultures (left) and from clusters (right), cultured as high density cell pellets for 0, 6 or 18 hours before replating. No differences in the number of neurons in outgrowth cultures was detected, while the number of neurons in pelleted cluster cell cultures declined significantly by 6 hours or 18 hours. The number of cultures for each condition are noted at the bottom of the graph (*P<0.01 by Mann-Whitney U-test).

or to crest cells, the majority of the S2 cells was removed at the end of each day, leaving behind the crest cell outgrowth on the culture substratum. At the end of the 5-day culture period, neural tube outgrowths that were overlaid with Delta1-expressing S2 cells during the first or second day of culture produced a significantly lower proportion of neurons than outgrowths that were initially overlaid with control S2 cells (Fig. 6C,D; P<0.05). In contrast, exposure of crest cells to Delta1-expressing S2 cells on the third day of culture had no significant effect on the proportion of neurons compared to crest cells that had been exposed to parental S2 cells throughout the culture period (Fig. 6C,E,F).

Consistent with previously known consequences of Notch signaling, interaction with Delta1-expressing cells could reduce neurogenesis in the culture by causing the potentially neurogenic cells to express an alternate crest phenotype (see Artavanis-Tsakonas et al., 1999; Morrison et al., 2000; Wakamatsu et al., 2000). Unfortunately, this possibility could not be tested directly because the total number of cells in each outgrowth culture varies significantly and, consequently, changes in the absolute number of other (pigment and glial) cell types in treated cultures could not be meaningfully assessed. However, as suggested above, the alternative that loss of neurogenic potential might be due to the death of a subpopulation of neurogenic precursors can be directly assessed. Thus, if co-culture with Delta1-expressing cells leads to increased death of neurogenic crest-derived cells, we predicted that the presence of zVAD-fmk in these co-cultures would rescue neurogenesis within nascent neural crest cell populations, as previously shown (Figs 4B,C). The results of such co-culture experiments confirmed that neurogenesis is rescued when crest cells are cultured in the presence of zVAD-fmk during the first 24-hour interval with Delta1-expressing S2 cells (Fig. 6G; P<0.001), whereas the presence of zVAD-fmk has no effect on the proportion of neurons in crest populations co-cultured with Delta1-expressing S2 cells during the third day of culture (Fig. 6H). A parsimonious interpretation of these results, therefore, is that interaction with Delta1-expressing cells increases death of neurogenic precursors in the crest cell population if it occurs immediately, but not at later times after dispersal.

Finally, we have previously shown that crest cells that disperse immediately (derived from 24-hr outgrowth cultures) are not sensitive to contact-mediated increase in TUNEL or decrease in the proportion of neurons (see Fig. 1B,C; Fig. 5B). Since we demonstrated above that such cells can respond to Delta1-expressing cells during the first 24 hours, we wished to determine whether brief supplementary interactions with Delta1-expressing cells could affect cell death in crest populations that had been prevented from dispersing, but were then cultured at a cell density permissive for neurogenesis. Accordingly, we measured the proportion of TUNEL-labeled crest cells after a brief initial co-culture with either Delta1-expressing or control cells. Because dissociated crest cells are initially weakly adherent to the culture substratum, however, many of them were lost when the S2 cells were removed after only 6 hours, and during the subsequent TUNEL procedure. Therefore, to prevent the loss of the crest cell target population, we used an alternate strategy for exposing newly plated crest cells to Delta1-expressing cells. Thus, for this experiment, crest cells were cultured for 6 hr on a feeder layer of L-cells stably transfected with quail Delta1. After this brief co-culture period, the crest cells were recognized by their smaller size and their immunoreactivity with an antibody to the quail cell-specific marker, QCPN. In such co-cultures with Delta1-expressing L cells, approximately 25% of previously undispersed (cluster-derived) crest cells were TUNEL positive, whereas comparable crest populations cultured on a feeder layer of control (untransfected) L-cells contained significantly fewer TUNEL-positive cells (P<0.05; Fig. 7, clusters). In contrast, crest cells that had dispersed immediately (neural tube outgrowth populations) showed greatly reduced numbers of TUNEL-positive cells, which were not significantly affected by exposure to Delta1-expressing feeder layers (Fig. 7, outgrowth). Taken together, these results indicate that a brief exposure to Delta1-expressing cells is sufficient to increase cell death in crest cell populations that have not undergone initial dispersal, but that crest cells that have dispersed immediately are not sensitive to such brief exposure to Delta1-expressing cells.

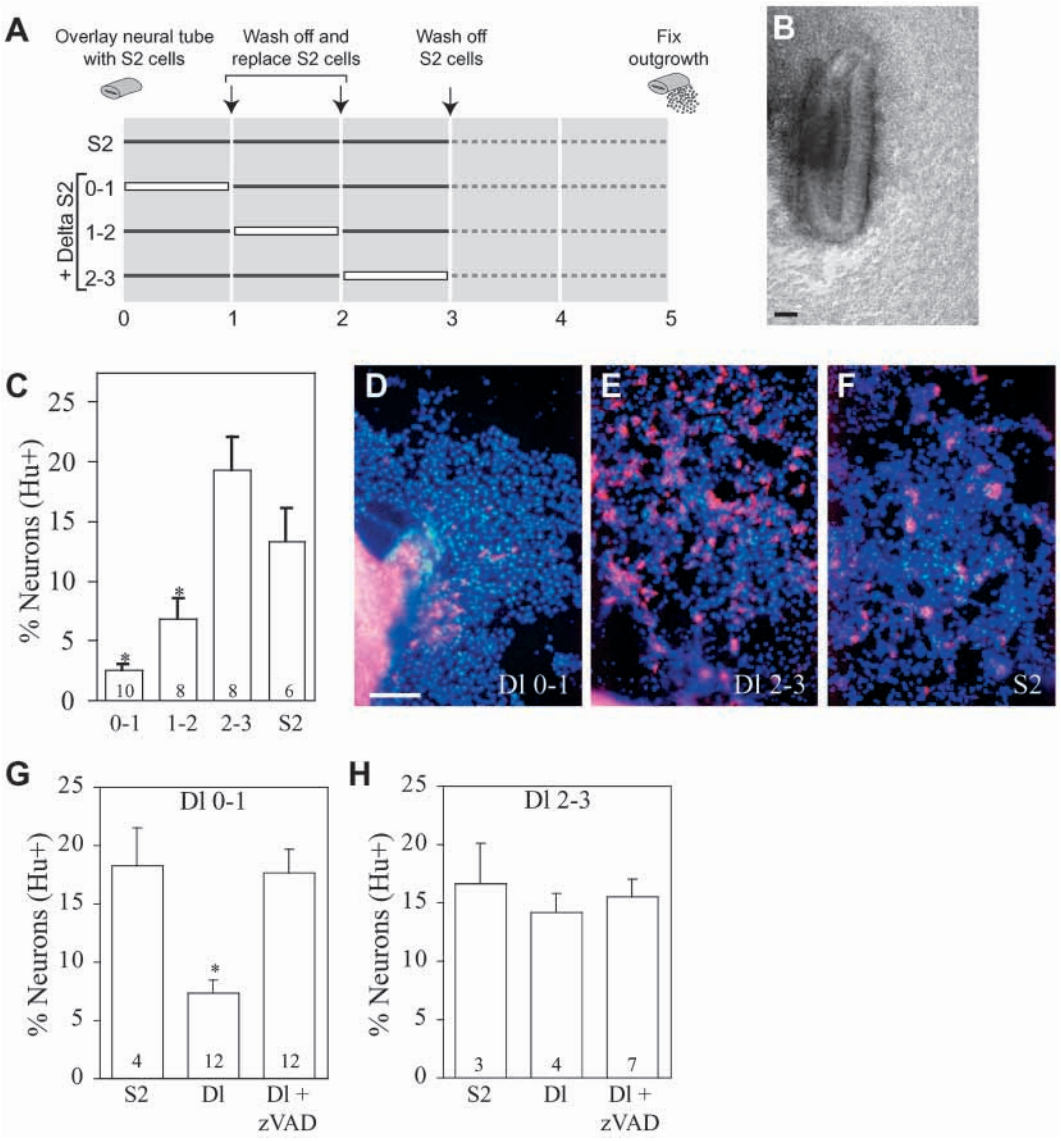

Fig. 6. Loss of neurogenic precursors in populations of outgrowth-derived crest cells depends on time of Delta-induced signaling. (A) Schematic showing timing of co-culture of neural crest cell outgrowth populations with Delta1-expressing (D1) or control S2 cells described in B and C. (B) Sample neural tube outgrowth after 24 hours, overlaid with Delta1-expressing S2 cells. (C) Crest cell outgrowths overlaid with Delta1-expressing S2 cells during the first day (0-1) show a lower proportion of neurons than control cultures (S2), or cultures overlaid with Delta1-expressing cells on the third day (2-3). (D-F) Representative outgrowths after 5 days of culture. In each case, the neural tube explant is located at the bottom left of each field. 16A11 staining of neurons is in red, DAPI staining of cell nuclei is in blue. Residual S2 cell bodies show up as small punctate spots in the DAPI staining. (G) Addition of zVAD-fmk (Dl+zVAD) prevents the decreased neurogenesis observed when cultures are overlaid with Delta1-expressing cells on the first day(Dl 0-1) (P<0.001). (H) zVAD-fmk treatment has no effect on cultures overlaid with Delta1-expressing cells on the third day of culture (Dl 2-3). Scale bar: 200 μm.

DISCUSSION

Previous studies (Vogel and Weston, 1988) have demonstrated that crest cell populations progressively and irreversibly lose neurogenic ability when they are prevented from dispersing in vitro. Recent work to assess the composition of the crest population that emerges from explanted avian trunk neural tubes suggests that most nascent crest cells are either bi-potent or fate-restricted neurogenic or gliagenic precursors (Henion and Weston, 1997). In such a heterogeneous mixture of cell types, it seems likely that developmentally relevant interactions can occur between and among various distinct cell types in the late pre-migratory and migrating populations. Such interactions would be likely to be diminished when crest cells disperse promptly, and promoted when such dispersal is delayed.

Our current results indicate that fate-restricted neurogenic precursors present in the initial crest population interact with each other or with bi-potent neuroglial precursors when dispersal is delayed, and that prolonged cell contact results in the selective elimination of these neurogenic populations. We cannot exclude the possibility that loss of neurogenic ability by the population is due to neurogenic cells adopting an alternate cell phenotype (see Morrison et al., 2000). However, in light of our caspase inhibitor results (which support the idea that loss of neurogenic ability is caused, at least in part, by the death of neurogenic precursors), fate-switching is unlikely to be the only mechanism involved. Thus, rescue of dying cells prevented loss of neurogenic ability by the crest cell population, even under conditions of cell contact that would normally promote loss of neurogenic ability of the population. Moreover, the fact that this loss of neurogenic ability is normally irreversible by subsequent cell dispersal (Vogel and Weston, 1988; present results), indicates that cells remaining in the crest population are unable to compensate for the loss of neurogenic cells, which, in turn, suggests that they too are developmentally restricted.

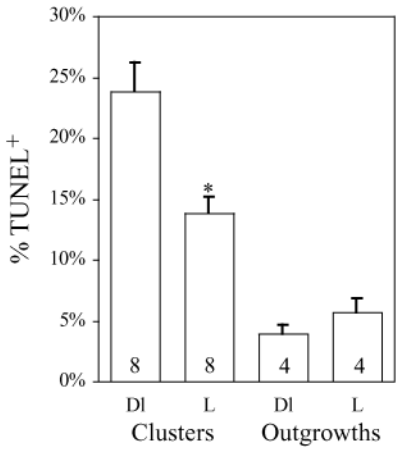

Fig. 7. The effect of Delta-induced signaling on the proportion of TUNEL-positive cells differs in co-cultures of undispersed (cluster-derived) and immediately dispersed (outgrowth-derived) crest cell populations. Significantly more dying cells were observed in cluster-derived cells than in dispersed cells co-cultured for 6 hours with Delta1-expressing L cells (*P<0.005).

Transient cell interactions in undispersed crest cell populations cause loss of neurogenic precursors

The ability to demonstrate that transient cell interactions within crest cell populations causes loss of neurogenic precursors is usually confounded by other environmental effects likely to be encountered by cells cultured at high densities. To address this issue, therefore, we designed our experiments to account for the alternate consequences of high cell density cultures, namely that such culture conditions deplete or condition the culture medium, or alter access or interactions with other components, such as matrix substrata, of the cellular environment. Thus, by co-culturing neurogenic populations in an environment conditioned by the presence of large numbers of other cells, or embedding cells at high density in collagen matrix so they do not aggregate, we were able to show that neither alterations (depletion or conditioning) of the nutrient medium, nor limited access to matrix substrata at high cell densities could account for the death of neurogenic precursors. Further, our experiments revealed that a transient initial period of cell-cell contact was sufficient to cause both cell death and reduced neurogenesis, even when the cells were subsequently cultured under standardized conditions known to permit neurogenesis. Importantly, the converse was also observed, namely, that neurogenesis by crest cell populations that had been permitted to disperse immediately was not adversely affected even by the high density culture conditions known to promote death of neurogenic precursors in populations that had not experienced an initial episode of dispersal.

Notch signaling may be involved in cell-contact mediated death of neurogenic precursors.

The Notch signaling pathway has been shown to affect the development of a wide range of cell types in both vertebrate and invertebrate embryos (Artavanis-Tsakonas et al., 1999; Campos-Ortega, 1995), and recently also to influence the course of apoptosis in some lineages (Miele and Osborne, 1999). We have shown that, unlike undispersed (cluster-derived) crest cell populations, cells that disperse immediately (neural tube outgrowths) do not exhibit decreased neurogenesis when cultured at high cell density. Consistent with the idea that Delta-Notch signaling is involved in this process, we have also shown that immediate exposure of nascent crest cell populations (neural tube outgrowth cultures) to Delta1-expressing cells results in decreased neurogenesis correlated with cell death (i.e. zVAD-fmk rescuable loss of neurogenesis). In contrast, delayed exposure of such neural tube outgrowth populations to Delta1-expressing cells (i.e. after the first 24 hours of culture) does not decrease neurogenic potential of the population. Likewise, we have shown that even a brief (6 hour) exposure of previously undispersed (cluster-derived) crest cell populations with Delta1-expressing cells is sufficient to increase cell death and correspondingly to decrease neurogenesis. These results suggest, therefore, that Notch signaling transiently mediates death of crest-derived neurogenic precursors in vitro.

The consequences of Notch signaling differ at different stages of crest cell development

Since Notch signaling is a dynamic process, it would be expected not only to affect a variety of cell types, but also to have different consequences within cell populations that subsequently arise during development from the initial population. In order to examine the effects of Notch signaling in detail, therefore, it is important to place Notch signaling in a temporal context. However, experimental perturbation of Notch signaling with appropriate temporal resolution is difficult to achieve by misexpression experiments (e.g. Chitnis et al., 1995; Coffman et al., 1993; Dornseifer et al., 1997; Wakamatsu et al., 2000), or by genetic alteration of signaling components in the Notch pathway. (e.g. Jiang et al., 1998; Xue et al., 1999). Unlike studies in which Notch signaling is activated or inactivated by misexpression or mutation, however, the present co-culture protocol permits more precise experimental control of the onset and duration of Notch signaling.

Based on the extensive literature on the role of Notch signaling, such a signaling pathway might be expected to cause competent precursors to express alternate crest cell fates at the expense of neuronal derivatives (see Artavanis-Tsakonis, et al., 1999; Morrison et al., 2000; Wakamatsu et al., 2000). However, among the cells of the early neural crest population, this mechanism would not account for the strong correlation between cell death and the loss of neurogenesis that was observed in our experiments. Likewise, such a mechanism could not easily account for the observation that zVAD-fmk, which specifically blocks developmentally regulated cell death, was able to rescue neurogenesis in interacting cell populations that had not undergone prompt initial dispersal.

The conclusion that Notch signaling can affect developmentally regulated cell death is not unprecedented, as Notch signaling has been shown to regulate cell survival in other systems (see Miller and Cagan, 1998; Miele and Osborne, 1999). It should be emphasized, however, that the consequences of Notch signaling in early crest populations appear to differ from that in older crest-derived populations. Thus, although Notch signaling appears to regulate the survival of neurogenic precursors in early crest populations, delayed exposure to Delta1-expressing cells, in crest cell populations

that had initially dispersed, neither adversely affects neurogenesis nor increases cell death. This inference is consistent with the more conventional, but equally important, role of Notch signaling that we have reported to occur in nascent DRG (see below; Wakamatsu et al., 2000), or in neural crest-derived stem cells isolated from rat sciatic nerve (Morrison et al., 2000).

Cell death may eliminate neurogenic crest-derived cells that fail to undergo prompt dispersal in vivo

Although the results of the present study were based solely on an in vitro model system, they nevertheless provide a potentially important, and testable, new insight about the role(s) that cell interactions play in premigratory and early migrating crest cell populations in vivo. We know, for example, that neural crest cell subpopulations normally disperse at different times along specific migration pathways. Crest cells that disperse immediately from the MSA on the ventromedial migration pathway produce neurons and glia in peripheral ganglia, whereas cells that undergo delayed dispersal from the MSA have limited neurogenic potential. The present results suggest that dispersal in vivo is not only essential for crest cells to reach their target destinations, but also may be required to permit the prolonged survival of a specific neurogenic subpopulation of neural crest cells. Thus, cell interactions involving Delta1-mediated Notch signaling might selectively 'edit' neurogenic precursors from crest cell populations that do not undergo prompt migration on the lateral pathway. We have reported that many migrating crest cells express Notch1, and a few express Delta1 (Wakamatsu et al., 2000). We suggest, therefore, that the loss of neurogenic ability in vivo could be due to cell-cell interactions among late migrating cells in or near the MSA. It is important to acknowledge, however, that we do not yet know the normal fate of all of the Notch- or Delta-expressing cells in the early crest population in vivo. Additional experiments, beyond the scope of the present paper, will be required to address these issues.

The conclusion that contact-mediated interactions cause the loss of neurogenic cells in the early crest cell population appears paradoxical since it is manifestly clear that neurogenesis does occur when crest-derived cells later coalesce and interact during gangliogenesis (Wakamatsu et al., 2000). Some insight into this apparent paradox may be provided by other published observations. Thus, it is well known that neural crest cells on the ventromedial pathway preferentially migrate in the rostral portion and avoid the caudal portion of each somite (Bronner-Fraser, 1986; Keynes and Stern, 1984; Loring and Erickson, 1987; Serbedzija et al., 1989; Teillet et al., 1987). The failure to migrate within the caudal half-somite is now believed to be due in part to the presence of the inhibitory secreted protein F-spondin (Debby-Brafman et al., 1999). It is noteworthy that when the caudal somite is replaced with migration-permissive rostral somite, or when F-spondin activity is blocked with antibody treatment, the crest cells migrating on the ventromedial pathway form larger than normal ganglia (Debby-Brafman et al., 1999; Gvirtzman et al., 1992; Kalcheim and Teillet, 1989). Conversely, when migration-permissive rostral somite is replaced with caudal somite, or when ectopic F-spondin is present, only small 'pseudo-ganglia' are formed adjacent to the dorsal neural tube. Although the change in ganglionic size could be due to many factors, one contributing factor to the size of the ganglia must certainly be differences in the number of neurons.

If prompt dispersal is required for the survival of neurogenic crest cells during embryogenesis, then increased cell death might be expected within the regions that are less favorable to dispersal. In fact, increased TUNEL is observed along the rostral edge of the caudal half of each somite of whole-mount avian embryos (Jeffs and Osmond, 1992; T. M. M., unpublished), where cell dispersal is restricted. We have also noted an increase in TUNEL-positive cells in the caudal half-somite resulting when transplanted CM-DiI-labeled neurogenic crest populations mislocalize in the caudal half-somite (Y. W., unpublished; Wakamatsu et al., 1998). We suggest, therefore, that delaying or preventing crest cell dispersal on the ventromedial pathway in vivo adversely affects the survival of the neurogenic crest population. Although observations concerning cell death within the crest populations of such experimental embryos have not yet been reported, we predict that embryonic regions that contain only caudal somite, or that overexpress F-spondin, would show elevated cell death of neurogenic precursors during initial crest migration.

Finally, although we have suggested that Notch signaling may play a role in cell interaction-mediated cell death, it is important to re-emphasize that this effect of Notch signaling on neurogenic crest cells must be transient. In fact, recalling that crest cells that migrate on the ventromedial pathway in vivo will normally aggregate to form the sensory and autonomic ganglia, and that Notch signaling also plays a role in regulating neurogenesis and gliogenesis in nascent DRG (Morrison et al., 2000; Wakamatsu et al., 2000), we suggest that the 'readout' of Notch signaling in crest-derived cells changes during the course of development. Thus, later interaction among crest-derived cells no longer adversely affects survival of neurogenic precursors, but rather mediates a developmental choice by neuroglial precursors. Prompt dispersal on the ventromedial migration pathway, therefore, may allow an early subpopulation of fate-restricted neurogenic cells, which is sensitive to contact-mediated cell death, to evade this process. However, subsequent contact-mediated interactions could still determine the fate of bi-potent neuroglial precursor cells (Henion and Weston, 1997; Morrison et al., 2000; Wakamatsu et al., 2000). Such a change in the readout of contact-mediated signaling is consistent with our observation that the effects of cell-cell contact or Delta-mediated signaling on neurogenic cells are diminished in crest cell populations (i.e. cells from outgrowth cultures) that had previously dispersed in vitro.

We thank Drs. Judith Eisen and Paul Henion for critical reading and helpful suggestions on the manuscript. This work was supported by Grants NS29438 and HD36396 to J. A. W. T. M. M. was supported in part by Grant 5-T32-HD07348 from NICHHD.

REFERENCES

Altshuler, D. and Cepko, C. (1992). A temporally regulated, diffusible activity is required for rod photoreceptor development in vitro. *Development* **114**, 947-957.

Anderson, D. J. (1993). Cell and molecular biology of neural crest cell lineage diversification. *Curr Opin Neurobiol.* **3**, 8-13.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch

signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Austin, C., Feldman, D., Ida, J. and Cepko, C. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121**, 3637-3650.

Baroffio, A., Dupin, E. and Le Douarin, N. M. (1988). Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc. Natl. Acad. Sci. USA* **85**, 5325-5329.

Bronner-Fraser, M. (1986). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev. Biol.* **115**, 44-55.

Bronner-Fraser, M. (1993). Segregation of cell lineage in the neural crest. *Curr. Opin. Genet. Dev.* **3**, 641-647.

Calof, A. L. (1995). Intrinsic and extrinsic factors regulating vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **5**, 19-27.

Campos-Ortega, J. A. (1995). Genetic mechanisms of early neurogenesis in *Drosophila melanogaster*. *Mol. Neurobiol.* **10**, 75-89.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene Delta. *Nature* **375**, 761-766.

Coffman, C. R., Skoglund, P., Harris, W. A., and Kintner, C. R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in *Xenopus* embryos. *Cell* **73**, 659-671.

Debby-Brafman, A., Burstyn-Cohen, T., Klar, A. and Kalcheim, C. (1999). F-Spondin, expressed in somite regions avoided by neural crest cells, mediates inhibition of distinct somite domains to neural crest migration. *Neuron* **22**, 475-488.

Dornseifer, P., Takke, C., and Campos-Ortega, J. A. (1997). Overexpression of a zebrafish homologue of the *Drosophila* neurogenic gene Delta perturbs differentiation of primary neurons and somite development. *Mech. Dev.* **63,** 159-171.

Erickson, C. A. and Goins, T. L. (1995). Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes. *Development* **121**, 915-924.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-34.

Fisher, L. and Van Belle, G. (1993). *Biostatistics: A Methodology for the Health Sciences*. New York: Wiley.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.

Glimelius, B. and Weston, J. A. (1981). Analysis of developmentally homogeneous neural crest cell populations in vitro. III. Role of culture environment in cluster formation and differentiation. *Cell Differ.* **10**, 57-67.

Gold, R., Schmied, M., Rothe, G., Zischler, H., Breitschopf, H., Wekerle, H. and Lassmann, H. (1993). Detection of DNA fragmentation in apoptosis: application of in situ nick translation to cell culture systems and tissue sections. *J. Histochem. Cytochem.* **41**, 1023-1030.

Gvirtzman, G., Goldstein, R. S. and Kalcheim, C. (1992). A positive correlation between permissiveness of mesoderm to neural crest migration and early DRG growth. *J. Neurobiol.* **23**, 205-216.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Henion, P. D. and Weston, J. A. (1994). Retinoic acid selectively promotes the survival and proliferation of neurogenic precursors in cultured neural crest cell populations. *Dev. Biol.* **161**, 243-250.

Henion, P. D. and Weston, J. A. (1997). Timing and pattern of cell fate restrictions in the neural crest lineage. *Development* **124**, 4351-4359.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375,** 787-790.

Jacobsen, M. D., Weil, M. and Raff, M. C. (1996). Role of Ced-3/ICE-family proteases in staurosporine-induced programmed cell death. *J. Cell Biol*.**133**, 1041-1051.

Jeffs, P. and Osmond, M. (1992). A segmented pattern of cell death during development of the chick embryo. *Anat. Embryol.* **185**, 589-598.

Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G., and Gridley, T. (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* **12,** 1046-1057.

Kalcheim, C. and Teillet, M. A. (1989). Consequences of somite manipulation on the pattern of dorsal root ganglion development. *Development* **106**, 85-93.

Keynes, R. J. and Stern, C. D. (1984). Segmentation in the vertebrate nervous system. *Nature* **310**, 786-789.

Krasnow, M. A., Saffman, E. E., Kornfeld, K., and Hogness, D. S. (1989). Transcriptional activation and repression by Ultrabithorax proteins in cultured *Drosophila* cells. *Cell* **57,** 1031-1043.

Le Douarin, N. and Kalcheim, C. (1999). *The Neural Crest.* 2nd edn. Cambridge: Cambridge University Press.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-917.

Loring, J., Glimelius, B., Erickson, C. and Weston, J. A. (1981). Analysis of developmentally homogeneous neural crest cell populations in vitro. I. Formation, morphology and differentiative behavior. *Dev. Biol.* **82**, 86-94.

Loring, J. F. and Erickson, C. A. (1987). Neural crest cell migratory pathways in the trunk of the chick embryo. *Dev. Biol.* **121**, 220-236.

Marusich, M. F., Furneaux, H. M., Henion, P. D. and Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25**, 143-155.

Miele, L. and Osborne, B. (1999). Arbiter of differentiation and death: Notch signaling meets apoptosis. *J Cell Physiol* **181**, 393-409.

Miller, D. T. and Cagan, R. L. (1998). Local induction of patterning and programmed cell death in the developing Drosophila retina. *Development* **125**, 2327-2335.

Morrison, S. J., Perez, S. E., Zhou Qiao, Verdi, J. M., Hicks, C., Weinmaster, G. and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.

Raff, M. C., Barres, B. A., Burne, J. F., Coles, H. S., Ishizaki, Y. and Jacobson, M. D. (1993). Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695-700.

Schneider, I. (1972). Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **27**, 353-365.

Serbedzija, G. N., Bronner-Fraser, M. and Fraser, S. E. (1989). A vital dye analysis of the timing and pathways of avian trunk neural crest cell migration. *Development* **106**, 809-816.

Sieber-Blum, M. (1989). Commitment of neural crest cells to the sensory neuron lineage. *Science* **243**, 1608-1611.

Sieber-Blum, M. (1999). The neural crest colony assay: Assessing molecular influences on development in culture. In *The Neuron in Tissue Culture* (ed. L. W. Haynes). pp. 5-22, New York: Wiley.

Teillet, M. A., Kalcheim, C. and Le Douarin, N. M. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. *Dev. Biol.* **120**, 329-347.

Vogel, K. S. and Weston, J. A. (1988). A subpopulation of cultured avian neural crest cells has transient neurogenic potential. *Neuron* **1**, 569-577.

Wakamatsu, Y., Mochii, M., Vogel, K. S. and Weston, J. A. (1998). Avian neural crest-derived neurogenic precursors undergo apoptosis on the lateral migration pathway. *Development* **125**, 4205-4213.

Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-2821.

Wakamatsu, Y. and Weston, J. A. (1997). Sequential expression and role of Hu RNA-binding proteins during neurogenesis. *Development* **124**, 3449-3460.

Wehrle-Haller, B. and Weston, J. A. (1997). Receptor tyrosine kinase-dependent neural crest migration in response to differentially localized growth factors. *BioEssays* **19**, 337-345.

Weil, M., Jacobson, M. D., Coles, H. S., Davies, T. J., Gardner, R. L., Raff, K. D. and Raff, M. C. (1996). Constitutive expression of the machinery for programmed cell death. *J. Cell Biol.* **133**, 1053-1059.

Weston, J. A. (1991). Sequential segregation and fate of developmentally restricted intermediate cell populations in the neural crest lineage. *Curr. Top. Dev. Biol.* **25**, 133-153.

Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum. Mol. Genet.* **8**, 723-730.