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Authors

Holcomb, J David
Mumm, Jeffrey S
Calof, Anne L

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Apoptosis in the Neuronal Lineage of the Mouse Olfactory Epithelium: Regulation *in Vivo* and *in Vitro*

J. David Holcomb,* Jeffrey S. Mumm,* and Anne L. Calof*,†,[1]

**Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242; and*
†*Department of Anatomy and Neurobiology and Developmental Biology Center,*
University of California, Irvine, California 92717

The olfactory epithelium (OE) of the mouse provides a unique system for understanding how cell birth and cell death interact to regulate neuron number during development and regeneration. We have examined cell death in the OE in normal adult mice; in adult mice subjected to unilateral olfactory bulbectomy (surgical removal of one olfactory bulb, the synaptic target of olfactory receptor neurons (ORNs) of the OE); and in primary cell cultures derived from embryonic mouse OE. *In vivo,* cells at all stages in the neuronal lineage—proliferating neuronal precursors, immature ORNs, and mature ORNs—displayed signs of apoptotic cell death; nonneuronal cells did not. Bulbectomy dramatically increased the number of apoptotic cells in the OE on the bulbectomized side. Shortly following bulbectomy, increased cell death involved neuronal cells of all stages. Later, cell death remained persistently elevated, but this was due to increased apoptosis by mature ORNs alone. *In vitro,* apoptotic death of both ORNs and their precursors could be inhibited by agents that prevent apoptosis in other cells: aurintricarboxylic acid (ATA), a membrane-permeant analog of cyclic AMP (CPT-cAMP), and certain members of the neurotrophin family of growth factors (brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 5), although no neurotrophin was as effective at promoting survival as ATA or CPT-cAMP. Consistent with observed effects of neurotrophins, immunohistochemistry localized the neurotrophin receptors trkB and trkC to fractions of ORNs scattered throughout neonatal OE. These results suggest that apoptosis may regulate neuronal number in the OE at multiple stages in the neuronal lineage and that multiple factors—potentially including certain neurotrophins—may be involved in this process.

INTRODUCTION

During vertebrate development, over half of the neurons in some areas of the nervous system die (Oppenheim, 1991). Naturally occurring neuronal death is thought to result from limitations in availability of trophic factors necessary for neuronal survival. Such factors appear to suppress an endogenous genetic program, known as programmed cell death or apoptosis (Oppenheim, 1991; Johnson and Deckwerth, 1993). *In vitro* studies indicate that the death of neurons following withdrawal of trophic factors displays morphological and biochemical hallmarks of apoptosis, including a requirement for new gene transcription and protein synthesis (e.g., Martin *et al.*, 1988; Scott and Davies, 1990; Edwards and Tolkovsky, 1994).

After development is completed, neuronal death may also be induced by axotomy or removal of synaptic targets. The fraction of a population of mature neurons that dies under these conditions often differs substantially from the fraction that dies during development: It varies widely depending on the type of neuron, and it is strongly dependent on both the age of the animal and the distance of the lesion from the neuronal cell body (Snider *et al.*, 1992). It is not yet clear whether injury-induced neuronal death results from a loss of trophic factors derived from synaptic target tissue or whether apoptosis is the predominant mechanism by which such cells die.

The mammalian olfactory epithelium (OE) provides a unique opportunity to study, in a single experimental prepa-

[1] To whom correspondence should be addressed at Dept. of Anatomy and Neurobiology, 364 Med Surge II, University of California College of Medicine, Irvine, California 92717-1275. Fax: (714) 824-1104. email: alcalof@uci.edu.

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ration, both target-dependent survival of developing neurons and lesion-induced neuronal death. Briefly, the olfactory receptor neurons (ORNs) of the OE, which innervate the olfactory bulb of the brain, undergo a slow process of turnover and replacement by newly generated neurons throughout adult life (Graziadei and Monti Graziadei, 1979). However, when one olfactory bulb is removed from an adult animal (a process known as unilateral bulbectomy), nearly all ORNs in the ipsilateral OE die (Costanzo and Graziadei, 1983). As ORNs die and the OE degenerates (decreases in thickness), cells in the basal compartment of the OE increase their proliferation and many lost ORNs are replaced (Schwartz Levey *et al.*, 1991). However, the OE never reaches its original thickness, apparently because the newly generated ORNs survive only a short time (Schwob *et al.*, 1992; Carr and Farbman, 1992, 1993). The rate of new ORN production appears to remain permanently elevated following removal of the olfactory bulb (Carr and Farbman, 1992), suggesting that proliferation of ORN precursors is linked to ongoing ORN death.

In this report, we examine cell death in the OE of the mouse under four conditions, three *in vivo* and one *in vitro:* In adult animals, cell death associated with acute and chronic responses to unilateral bulbectomy is compared to that in unlesioned animals; in culture, the process of cell death is examined in neuronal cells isolated from embryonic OE. In each case, we identify the types and developmental stages of cells that are dying, analyze the timecourse of cell death, and evaluate whether death exhibits characteristics of apoptosis. In addition, we use tissue culture of embryonic OE to determine which, if any, of the trophic factors of the neurotrophin family are sufficient to promote ORN survival. Our results indicate that, *in vivo* as well as *in vitro,* cells at multiple stages in the ORN lineage undergo cell death, and this death exhibits definitive characteristics of apoptosis. Interestingly, the data suggest that different factors may be responsible for regulating apoptosis at these different developmental stages.

MATERIALS AND METHODS

Materials

Recombinant human NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 5 (NT-5) were obtained from Genentech (generous gifts of David Shelton, Enrique Escandon, John Winslow, Karoly Nikolics, Gene Burton, and Arnon Rosenthal). Growth factors were stored at −85°C in 1 mg/ml Clinical Reagent Grade bovine serum albumin (ICN Biochemicals) in calcium- and magnesium-free Hank's balanced salt solution. [^{3}H]Thymidine ([^{3}H]TdR; 70–90 Ci/mmol) was from New England Nuclear. NTB2 emulsion, D-19 developer, and fixer were from Eastman Kodak. Tissue culture media and antibiotics were from Gibco BRL. Antibody to rat trkA ectodomain (amino terminal cysteine plus amino acids 322–346, CSVLNETSFIFTQFLESALTNETMRH: ''rtrkA.EX2''; Clary *et al.*, 1994) was the kind gift of Louis Reichardt, UC San Francisco. Affinity-purified antibody to rat trkB ectodomain (amino acids 23–36, AFPRLEPNSIDPEN: ''anti-trkB$_{23-36}$''; Yan *et al.*, 1994) was the kind gift of Stuart Feinstein and Monte Radeke, UC Santa Barbara. Affinity-purified antibody to trkB intracellular domain (amino acids 794–808, HTRKNIKSIHTLLQN: ''anti-trkB (794)'') was from Santa Cruz Biotechnology (the sequence this antibody is directed against is almost completely divergent in the corresponding regions of trkA and trkC; therefore, this antibody should be specific for trkB). Antiserum to trkC intracellular domain and the peptide immunogen used to produce it (amino acids 637–653, MILVDGQPRQAKGELGL: ''anti-trkCin2''; Hoehner *et al.*, 1995) were generous gifts from David Kaplan, NCI-Frederick. Unless noted, other reagents were from Sigma.

Surgery

For unilateral bulbectomy experiments, adult male mice (4–6 weeks old) of two strains were used: outbred Swiss CD-1s (Charles River) and OT-2 transgenics (CBA × C57/B16 background; the kind gift of Dr. Frank Margolis (Danciger *et al.*, 1989)). All mice were maintained on a 12-hr light/dark cycle, and surgeries were generally performed between 10 AM and 2 PM. Anesthesia was obtained via ip injection of ketamine (45 mg/kg) and pentobarbital (35 mg/kg) for CD-1 mice, and 0.2–0.4 ml Avertin (1.77 m*M* 2,2,2-tribromoethanol and 2.5% tert-amyl alcohol dissolved in water) for OT-2 mice. The region of the head above the olfactory bulb was shaved with clippers and antisepsis obtained with 70% ethanol. Mice were held in place with a Stoelting stereotaxic apparatus with mouse adapter. After a midline incision through the scalp, a Dremel drill with diamond-tipped burr was used to expose the left olfactory bulb, which was aspirated with a No. 3 Baron suction tube. Hemostasis was obtained with Gelfoam powder and the scalp was closed with a 6-O Ethilon suture. Mice were kept warm during recovery.

Tissue Culture

For culture of OE neuronal cells, tissue from both OT-2 and CD-1 strains was used (analysis of TUNEL staining at 24 hr in culture (see below) gave similar results for OE neuronal cells isolated from either strain, so the two strains were used interchangeably). Mice were naturally mated, with the day at which a vaginal plug was detected designated Day E0.5. OE neuronal cells (ORNs plus their immediate neuronal precursors, INPs) were prepared from suspension cultures of purified embryonic OE using published methods (Calof and Lander, 1991; Calof *et al.*, 1995) with minor modifications: turbinates were dissected from E16.5 to E17.5 mouse embryos, and suspension culture of purified

OE was for 6–8 hr. Following suspension culture, the neuronal cell fraction (ORNs + INPs) was isolated from horizontal basal cells and sustentacular cells by selective trituration, centrifugation, and filtration as described, except that no trypsin was used prior to the first trituration step, and 10-μm nylon mesh (CMN-10-D, Small Parts Inc.) was used for filtration. Composition of the OE neuronal cell fraction assessed 4 hr after plating was 76% ORNs (identified by expression of the neuronal cell adhesion molecule, NCAM; Calof and Chikaraishi, 1989) and 24% INPs (NCAM-negative; Calof and Lander, 1991). Viability was assessed by calcein AM staining and ethidium homodimer-1 exclusion (Live/Dead kit, Molecular Probes) and was >90% at the time of plating. Cells were cultured in serum-free low calcium (0.1 m*M* $CaCl_2$) culture medium containing 5 mg/ml crystalline bovine serum albumin (BSA) (LCCM; Calof and Lander, 1991). Tissue culture substrata were either 96-well tissue culture trays (Costar 3596) or 12-mm acid-cleaned glass coverslips (Propper; Calof and Chikaraishi, 1989) that had been treated with 1 mg/ml poly-D-lysine in water overnight at 4°C, then washed in water, and sterilized by ultraviolet light.

For primary culture of mouse thymocytes, thymic tissue harvested from neonatal mice was minced and thymocytes were separated from stroma by trituration followed by filtration through 20-μm nylon mesh (CMN-20-D, Small Parts Inc.). Thymocytes were cultured in RPMI 1640 containing 10% fetal bovine serum (HyClone), 2 m*M* L-glutamine (Gibco BRL), and penicillin–streptomycin (100 U/ml of each; Gibco BRL).

Immunocytochemistry, Autoradiography, and Detection of DNA Fragmentation

Tissue sections. For detection of DNA fragmentation and staining with all antibodies except for anti-trkB and anti-trkC, adult male mice were euthanized by lethal overdose of pentobarbital (ip). The entire nasal vault including cribriform plate was dissected and fixed by freeze-substitution as follows: 30 min in 2-methylbutane chilled on dry ice, 3 × 24 hr in 100% ethanol equilibrated to −80°C, 24 hr in 100% ethanol at −20°C, 24 hr in 95% ethanol at −20°C. Tissue was rehydrated at 4°C with 1-hr rinses in 95 and 70% ethanol, followed by a rinse in phosphate-buffered saline (PBS). Decalcification was carried out over 7 days at 4°C in 390 m*M* EDTA, pH 7.1 (modified from Mori *et al.*, 1988). Following decalcification, tissue was equilibrated in 30% sucrose in PBS at 4°C and then sectioned at 12 μm in the transverse (horizontal) plane using a Jung CM 3000 cryostat. Sections were collected on ProbeOn Plus slides (Fisher) and stored at −20°C.

For staining with anti-trkB (794), heads of P1 OT-2 mice were immersed for 3 hr in Bouin's fluid (Brown, 1978), dehydrated through xylenes, embedded in paraffin, and sectioned at 10 μm. Sections were stored at room temperature. For staining with anti-trkB$_{23-36}$ and anti-trkCin2, heads of P0–P1 OT-2 mice were fixed by immersion for 3 hr in 4% paraformaldehyde in 0.02 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, and then equilibrated in 15% sucrose/7.5% gelatin and sectioned at 12 μm on a cryostat. Sections were stored at −20°C.

Detection of DNA fragmentation. Tissue sections were air-dried, rehydrated with PBS for 10 min, and permeabilized in 0.1% Triton X-100 in PBS for 1 hr (30 min for double-labeling experiments). Sections were rinsed twice in TDT buffer (30 m*M* Tris–HCl, 140 m*M* sodium cacodylate, 1 m*M* cobalt chloride, pH 7.2) for 5 min each at room temperature before end-labeling of DNA fragments with biotinylated dUTP (TUNEL staining; Gavrieli *et al.*, 1992) as follows: ∼100 μl of reaction mixture (20 μ*M* dUTP and biotin-16-dUTP in a 2:1 ratio in TDT buffer plus 0.2 U/ml terminal transferase (all Boehringer-Mannheim)) was placed onto the sections and incubated for 1 hr at 37°C. The reaction was stopped by rinsing in TB buffer (300 m*M* NaCl, 30 m*M* sodium citrate) for 15 min. Sections were blocked with 10 mg/ml crystalline BSA (ICN Biochemicals) in PBS, and biotinylated DNA was detected with either Texas red- or FITC-conjugated Z-avidin (Zymed) diluted 1:50 in 1 mg/ml crystalline BSA in PBS.

For *in vitro* TUNEL staining, cultures were fixed for 1 hr in 10% formalin/5% sucrose/PBS at room temperature, permeabilized in PBS + 0.1% Triton X-100, and end-labeled with biotin-dUTP as described above. For cultures grown in 96-well tissue culture trays, biotinylated DNA was detected using horseradish peroxidase-conjugated Z-avidin (1:1000 in 1 mg/ml crystalline BSA in PBS; Zymed) using 3-amino-9-ethylcarbazole as the chromagen. For cultures grown on glass coverslips, biotinylated DNA was detected using fluorescent avidins as described above.

Immunocytochemistry. Total postmitotic ORNs were detected using H28 monoclonal rat anti-NCAM (kind gift of Christo Goridis) detected with Texas red-conjugated goat anti-rat IgG (1:50 dilution; Jackson Immunoresearch) (DeHamer *et al.*, 1994). In OT-2 transgenic mice, Thy1.1 protein is expressed exclusively in OMP^+ cells (Danciger *et al.*, 1989). Therefore, mature ORNs were detected using a mouse monoclonal anti-Thy1.1 antibody (mAb 22, 1:500 dilution of ascites fluid; Greenspan and O'Brien, 1989), visualized with Texas red-conjugated goat anti-mouse IgG1 (1:50 dilution; Southern Biotechnology). Horizontal basal cells were stained with rabbit antiserum to keratins (Dakopatts Z622; diluted 1:400) and detected with Texas red-conjugated goat anti-rabbit IgG (1:50 dilution; Jackson Immunoresearch). For staining with anti-trkB (794), sections were deparaffinized through xylenes and rehydrated in PBS and antibody was applied at 5 μg/ml overnight at 4°C. Primary antibody was detected using biotinylated goat-anti-rabbit IgG (2.5 μg/ml; Vector) and Texas red-conjugated streptavidin (1:1000 dilution; Gibco BRL). For staining with anti-trkB$_{23-36}$ (10 μg/ml) and anti-trkCin2 (1:200 dilution of rabbit antiserum), sections were air-dried and permeabilized in PBS + 0.1% Triton X-100, antibodies were applied for 2.5

hr at room temperature, and primary antibody was detected using Cy3-conjugated donkey anti-rabbit IgG (1:100 dilution; Jackson Immunoresearch). Immunocytochemistry on cultured cells was performed using the same reagents, with cells that had been cultured on glass coverslips and fixed for 10 min in 10% formalin/PBS/5% sucrose.

Autoradiography. For *in vivo* studies of apoptosis in ORN precursors, adult male OT-2 and CD-1 mice were subjected to unilateral bulbectomy and given two sequential injections (each 20 μCi per g body wt) of [^{3}H]TdR at 2-hr intervals prior to sacrifice at 24 hr postsurgery. OE tissue was dissected, fixed, and sectioned as described above. Slides were processed for immunocytochemistry and TUNEL staining as described above, then dipped in NTB2 emulsion (diluted 1:1 in water), exposed for 48 hr at -80°C, and developed with D-19 developer.

For *in vitro* studies of apoptosis in ORN precursors, the dissociated olfactory neuronal cell fraction was plated on polylysine-coated glass coverslips in LCCM. After a 20-hr incubation, cultures were pulsed for 4 hr with 2.5 μCi/ml [^{3}H]TdR and then fixed and processed for TUNEL and NCAM immunoreactivity. Coverslips were dipped, exposed, and developed in the same manner as tissue sections.

RESULTS

OE Cells Induced to Die in Vivo Undergo DNA Fragmentation

Fragmentation of nuclear DNA is, in many cell types, a hallmark of apoptosis (Arends and Wyllie, 1991; Gavrieli *et al.*, 1992; Deckwerth and Johnson, 1993). The TUNEL technique (DNA end-labeling with deoxynucleotide terminal transferase and dUTP-biotin (Gavrieli *et al.*, 1992)) was used to test for DNA fragmentation in the mouse OE following unilateral bulbectomy. As shown in Fig. 1A, within one day following bulbectomy, many cells in the OE on the operated side were TUNEL$^+$ (had fragmented DNA). At the same time, few cells in the contralateral OE of the same animal were TUNEL$^+$ (Fig. 1B). An elevation in the number of TUNEL$^+$ cells in the OE on the operated, versus contralateral, side persisted for at least 8 weeks following surgery (Figs. 1C and 1D).

In order to assess the timecourse and extent of cell death in the OE following disruption of contact with the OB, sections of OE from animals sacrificed at timepoints from 12 hr to 12 weeks following bulbectomy were processed for TUNEL staining. Unoperated (control) animals were also evaluated. In OE on the operated side (Fig. 2A), the number of TUNEL$^+$ cells increased sharply by 12 hr postsurgery and peaked at 2 days; it then declined rapidly over the next 24 hr, reaching a minimum 5 days following bulbectomy. Thereafter, the number of TUNEL$^+$ cells remained relatively low, but still higher than in the contralateral OE. The extent of TUNEL staining in the contralateral OE (Fig. 2B) approximated that observed in the OE of unoperated (control) animals (Fig. 2B, $t = 0$) throughout the entire postoperative period.

The individual points in Figs. 2A and 2B show data from single sections, to convey a sense of section-to-section and interanimal variability. The figure includes data from two mouse strains: CD-1 (an outbred albino; solid squares) and the transgenic strain OT-2 (see below; open squares). Since results did not differ substantially between the two strains (analysis of variance at each timepoint at which both strains were analyzed revealed no significant differences), data from both were pooled and used to construct a single set of curves showing changes in mean numbers of TUNEL$^+$ cells in the operated, versus contralateral, OE as a function of time following unilateral bulbectomy (Fig. 2C). Figure 2C also correlates these data with measurements of mean thickness of OE on the operated side (thickness is an indicator of cell number; Costanzo and Graziadei, 1983; Schwartz-Levey *et al.*, 1991).

Cell Types That Undergo Induced Death

The adult OE contains several different cell types: ORNs, precursors of ORNs, supporting or sustentacular cells, and horizontal basal cells. These cells can be identified by their laminar position within the epithelium and their expression of specific markers: The cell bodies of sustentacular cells form a single layer in the apical OE. The cell bodies of mature ORNs, cells that uniquely express olfactory marker protein (OMP), are located below the sustentacular cells in the middle half of the epithelium (Margolis, 1980; Verhaagen *et al.*, 1990; Schwob *et al.*, 1992). Immature postmitotic ORNs lie deep to mature ORNs; they lack OMP but, along with mature ORNs, express the neural cell adhesion molecule NCAM (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994). Deeper still are the actively dividing INPs (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994)—sometimes called ‘‘globose’’ basal cells—that give rise to ORNs. INPs are located in the basal one-fourth of the epithelium between ORNs and horizontal basal cells (Mackay-Sim and Kittel, 1991a; Schwartz Levey *et al.*, 1991; Caggiano *et al.*, 1994). They have so far been best characterized *in vitro,* where they are recognizable by the absence of markers for other OE cell types (they lack NCAM, OMP, and keratins) and by their ability to be labeled by short pulses of [^{3}H]TdR (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994). Horizontal basal cells may be identified by their expression of keratins and their close apposition to the basal lamina (Vollrath *et al.*, 1985; Calof and Chikaraishi, 1989); in the past these cells were postulated to be neuronal stem cells, but currently the idea that they are even in the ORN lineage is controversial (Harding *et al.*, 1977; Calof and Chikaraishi, 1989; Mackay-Sim and Kittel, 1991a; Guillemot *et al.*, 1993; Caggiano *et al.*, 1994).

To determine which of these cell types undergo cell death with accompanying DNA fragmentation following bulbec-

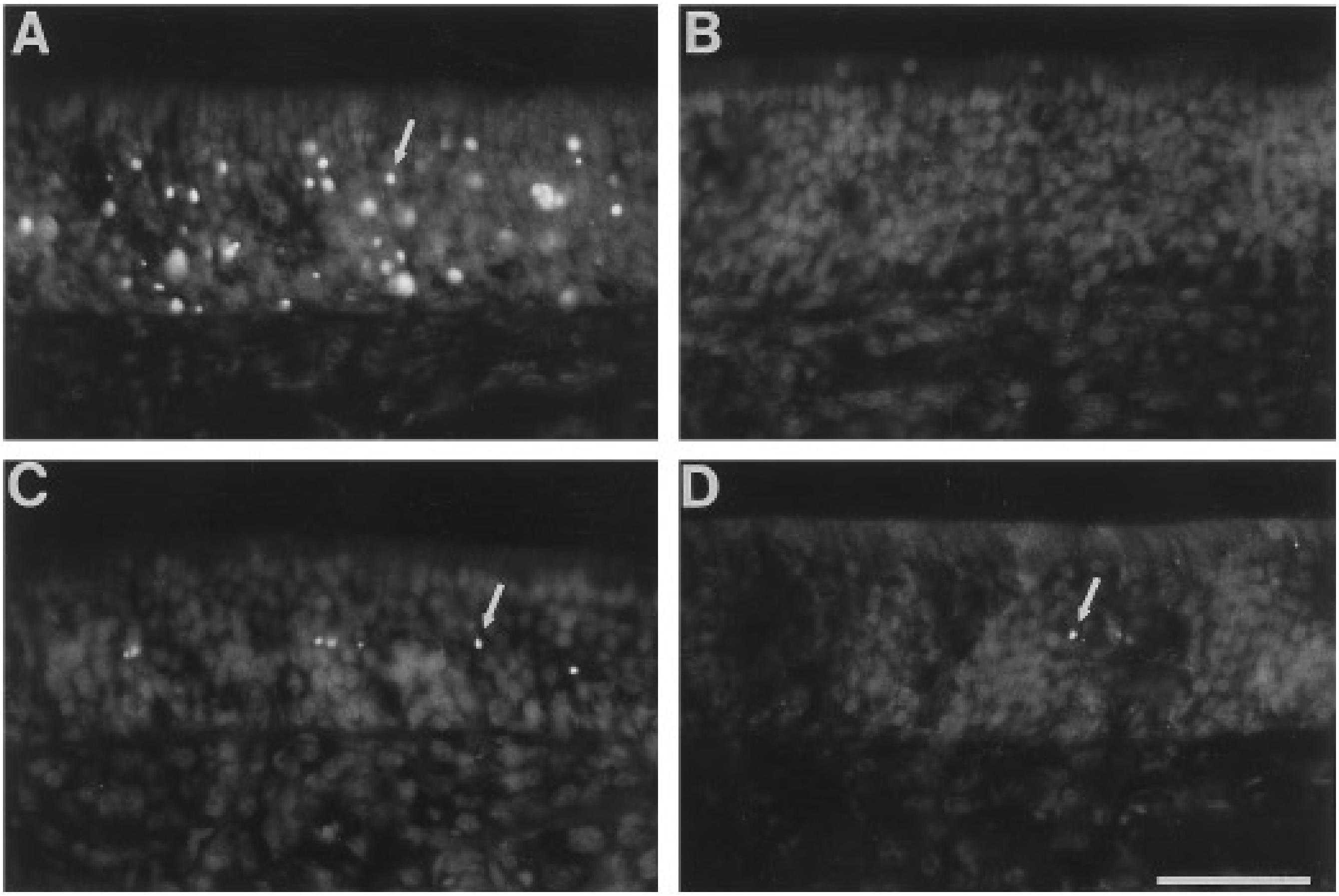

FIG. 1. Induced DNA fragmentation in the olfactory epithelium following unilateral bulbectomy. (A) OE from the bulbectomized (OBX) side of an animal sacrificed 1 day postsurgery. (B) OE from the contralateral (Cont) side, immediately opposite OE shown in A. (C) OBX OE from an animal sacrificed at 56 days postsurgery. (D) Cont OE immediately opposite OE shown in C. Arrows point to brightly fluorescent nuclei of TUNEL-labeled OE cells. Bar, 50 μm.

tomy, TUNEL labeling was combined with cell type-specific immunohistochemistry. Because the tissue processing required for the TUNEL reaction (see Materials and Methods) leads to poor preservation and/or retention of OMP, an alternative approach was used to identify mature (OMP-expressing) neurons: In OT-2 transgenic mice, OMP gene regulatory elements drive the expression of a Thy1.1 reporter gene. This gene product is expressed exclusively in OMP$^+$ cells (Danciger *et al.*, 1989) and can be detected with monoclonal anti-Thy1.1 antibodies.

TUNEL staining was combined with anti-NCAM, anti-Thy1.1, and anti-keratin immunohistochemistry in unoperated (control) animals and animals sacrificed at 1 or ⩾56 days following unilateral bulbectomy. Figure 3 shows typical sections of OE in which TUNEL staining was combined with anti-NCAM (Figs. 3A, 3C, and 3E) and anti-Thy1.1 (Figs. 3B, 3D, and 3F) immunohistochemistry. Numbers of TUNEL$^+$ cells that resided either within or outside the layers of keratin, NCAM, or Thy1.1 immunoreactivity were counted for each condition. The percentages of TUNEL$^+$ cells that were keratin$^-$, keratin$^+$, NCAM$^-$, NCAM$^+$, Thy1.1$^-$, or Thy1.1$^+$ were then calculated from these numbers.

In general, TUNEL$^+$ cells were restricted to layers expressing neuronal markers (Table 1). No TUNEL$^+$ nuclei were found among the keratin$^+$ horizontal basal cells nor in the sustentacular cell body layer (not shown). For example, 1 day following bulbectomy, 92% of TUNEL$^+$ cells on the OBX side were postmitotic ORNs: 73% of TUNEL$^+$ cells on the OBX side were mature ORNs (Thy1.1$^+$) and 19% were immature ORNs (determined by subtracting the percentage that was Thy1.1$^+$ from the percentage that was NCAM$^+$). The remaining 8% had the characteristics of INPs: they were NCAM$^-$ and located between the ORN and horizontal basal cell layers (see Fig. 3C, arrowhead).

To verify that at least some of these TUNEL$^+$, NCAM$^-$

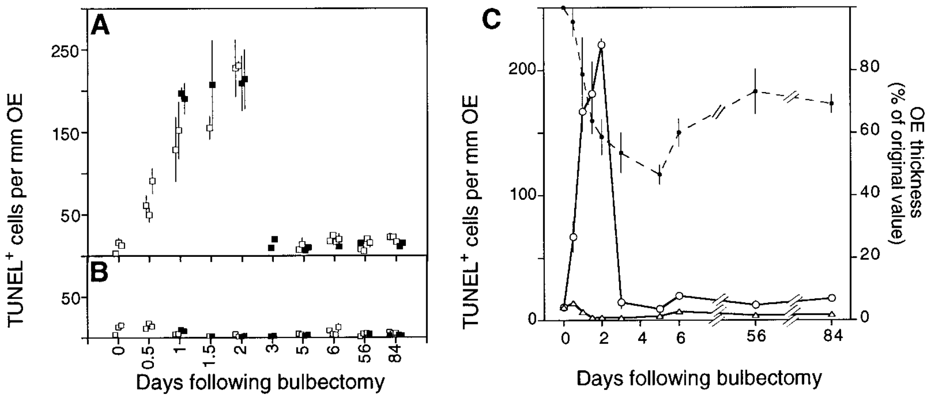

FIG. 2. Timecourse of DNA fragmentation following unilateral bulbectomy. Cryostat sections of OE from unoperated (control) and bulbectomized mice (sacrificed at postoperative timepoints indicated) were processed for TUNEL. TUNEL$^+$ cells were counted in OBX (A), Cont (B), and unoperated (A and B, Time = 0) septal OE. Each square represents the average number of TUNEL$^+$ cells/mm OE in a single histological section; error bars reflect SEM of multiple fields (5–10) of a single section. Analysis was restricted to horizontal sections taken at a similar dorsoventral level (~1.5 mm from the ventral extent of the OE) and to OE lining the posterior 2–3 mm of the nasal septum (in some cases, OE lining the endoturbinates was also analyzed, and gave similar results (not shown)). Sections from two mice were analyzed for each time point. Animals used in these experiments were either outbred CD-1 mice (solid squares) or OT-2 transgenic mice (open squares). (C) Pooled data from CD-1 and OT-2 mice. Mean numbers of TUNEL$^+$ cells/mm OE (±SEM) are plotted (open circles, OBX OE; open triangles, Cont OE), together with changes in the average thickness of the OBX OE (solid circles), over time following bulbectomy. Where error bars are not seen, the error was small enough to be obscured by the symbol representing the data point. Differences between OBX and Cont OE were statistically significant for all times ⩽2 and ⩾6 days ($P \leqslant 0.02$ except at 56 days, where $P = 0.055$; Student's t test; Glantz, 1992).

cells were INPs, animals were given two injections of [^{3}H]-TdR at 2-hr intervals prior to being sacrificed at 24 hr post-surgery, so that INPs in S-phase could be detected. TUNEL$^+$ cells were evaluated for the presence of [^{3}H]TdR incorporation in OE from both OBX and Cont sides; more than 75 mm of OE were counted in sections from four different animals. The results indicate that, although rare, TUNEL$^+$/[^{3}H]TdR$^+$ cells can be detected *in vivo:* 1.21% (±0.08% (SEM)) of [^{3}H]TdR$^+$ cells in the OBX OE (656 [^{3}H]TdR$^+$ cells counted) and 0.12% (±0.09% (SEM)) of [^{3}H]TdR$^+$ cells in the Cont OE (2113 [^{3}H]TdR$^+$ cells counted) were found to be TUNEL$^+$. These data indicate that ORN precursors undergo cell death with DNA fragmentation *in vivo* and suggest that the number of precursors undergoing cell death may be elevated during the acute response to bulbectomy (the difference between OBX and Cont sides was statistically significant: $P < 0.001$, Student's t test (Glantz, 1992)). An example of a TUNEL$^+$/[^{3}H]TdR$^+$ neuronal precursor in the OE on the OBX side of one of these animals is shown in Fig. 4.

At ⩾56 days following bulbectomy, the composition of TUNEL$^+$ cells in the OBX OE differed significantly from that at 24 hr. At this stage, all TUNEL$^+$ cells in the OBX OE were ORNs (NCAM$^+$). Furthermore, 91% of these were mature ORNs (Thy1.1$^+$). In the Cont OE, the composition of TUNEL$^+$ cells was similar to that in unoperated (control) OE at both short and long times following bulbectomy (Table 1).

Since the absolute numbers of TUNEL$^+$ cells differ substantially among the five conditions examined in Table 1 (unoperated control; short- and long-term survival, OBX and Cont sides), it is instructive to multiply the values in Table 1 by the mean numbers of TUNEL$^+$ cells found in the OE for each condition (values for OT-2 mice from Figs. 2A and 2B), in order to evaluate the magnitude of death for each cell type. The results of these calculations, shown graphically in Fig. 5, indicate the following: In normal OE (from the Cont side of short- or long-term bulbectomized animals or from Unop animals), there is a constitutive, low level of DNA fragmentation among all cell types of the neuronal lineage: mature ORNs (NCAM$^+$, Thy 1.1$^+$), immature ORNs (NCAM$^+$, Thy 1.1$^-$), and neuronal precursors (NCAM$^-$, keratin$^-$ cells in basal OE). Following bulbectomy, on the OBX side, there is a rapid increase in DNA fragmentation among *all* of these cell types. However, after a long survival time, only mature ORNs show an elevated level of DNA fragmentation compared to that in the Cont side. These results suggest that the causes of cell death in the OE in response to acute versus chronic bulbectomy may not be identical (see Discussion).

Apoptotic Death of Olfactory Neuronal Cells in Vitro

Previous work has shown that OE explants purified from E14.5 to E15.5 mouse embryos can be cultured in serum-

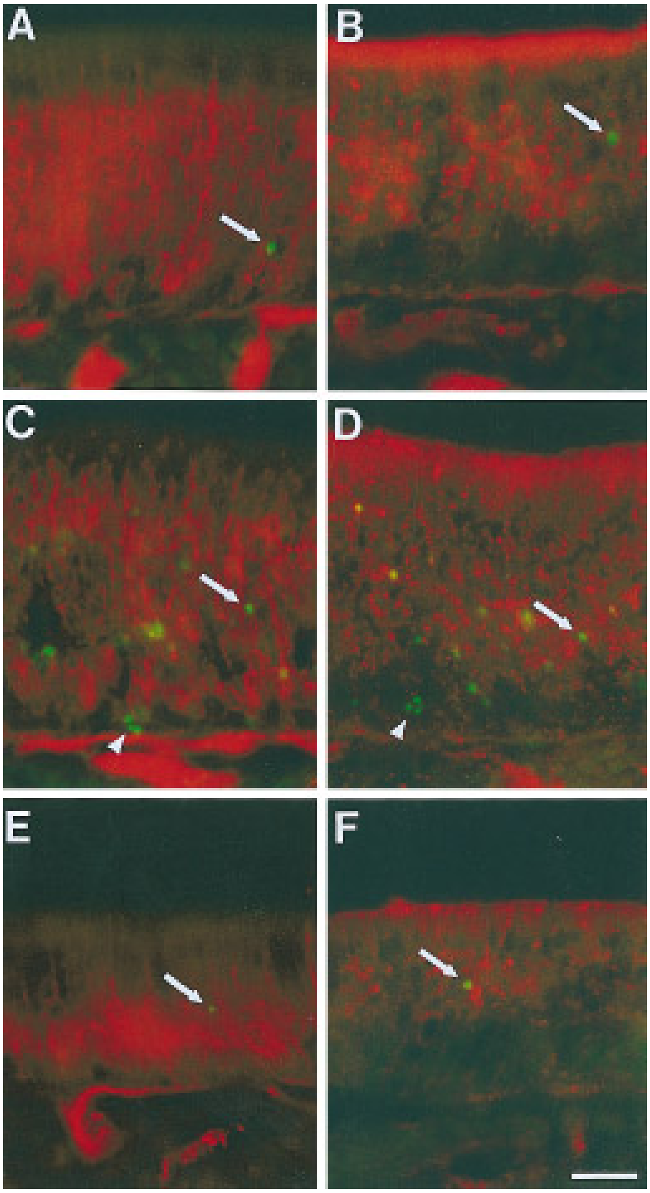

FIG. 3. Phenotypes of cells with DNA fragmentation in OE. Antibodies to NCAM and Thy1.1 (shown in red) were used in combination with TUNEL staining (shown in green) in order to define phenotypes of cells undergoing DNA fragmentation in sections of OE from unoperated (control) and bulbectomized OT-2 mice. Bulbectomized mice were sacrificed at 1 or 67 days after surgery. Double-exposure fluorescence photomicrographs are shown: (A) NCAM/TUNEL of OE from unoperated (control) animal; (B) Thy1.1/TUNEL of OE from the same animal as A. (C) NCAM/TUNEL of 1-day OBX OE; (D) Thy1.1/TUNEL of 1-day OBX OE from the same animal as C. In these two photomicrographs, some of the TUNEL$^+$ cells appear less bright than others because they are not in the plane of focus. (E) NCAM/TUNEL of 67-day OBX OE; and (F) Thy1.1/TUNEL of 67-day OBX OE from the same animal as E. Arrows indicate TUNEL$^+$ cells within the NCAM$^+$ (A, C, and E) or Thy1.1$^+$ (B, D, and F) cell layers. Arrowheads indicate TUNEL$^+$ cells lying below NCAM$^+$ and Thy1.1$^+$ cell layers (C and D, respectively). Note the overall decrease in thickness of the OE in the chronically bulbectomized animals (E and F). Bar, 20 μm.

free, defined medium and that INPs and ORNs in these cultures proliferate, differentiate, and survive for a short period of time. Within a week, however, the neuronal cells in such cultures die in the absence of added growth factors (Calof and Chikaraishi, 1989). Since explantation at this developmental stage severs already formed contacts between the OE and the developing olfactory bulb, it seemed possible that causes of death of OE neuronal cells *in vitro* might be similar to causes of OE neuronal cell death in adult animals following bulbectomy. To explore this possibility and to identify potential factors mediating ORN survival, the dissociated neuronal cell fraction from E16.5 to E17.5 OE suspension cultures was grown in minimally supplemented, defined medium (see Materials and Methods), and the TUNEL technique was used to identify cells undergoing DNA fragmentation.

Typical results are illustrated in Fig. 6. At the time of plating few (<5%) cells had TUNEL$^+$ nuclei (Fig. 6A), but after 24 hr in culture this number increased to ~50% (Fig. 6B). Interestingly, the majority of cells in culture at 24 hr were phase-bright and neurite-bearing (see also Fig. 7, below), consistent with the idea that DNA fragmentation precedes morphological changes associated with cell death. As a control for reliability of the TUNEL reaction *in vitro*, dexamethasone-induced DNA fragmentation in neonatal mouse thymocytes (Wyllie *et al.*, 1984) was also demonstrated (Figs. 6C and 6D).

To verify that DNA fragmentation by OE neuronal cells is indicative of apoptosis, pharmacological treatments known to prevent or delay apoptosis in other cell types were tested for their effects on these cultures. As shown in Table 2, actinomycin D, cycloheximide, and aurintricarboxylic acid (ATA) each reduced by ⩾50% the numbers of TUNEL$^+$ cells in 24-hr cultures of OE neuronal cells (in the case of ATA, inhibition of DNA fragmentation was confirmed by gel electrophoresis; data not shown). Moreover, ATA kept OE neuronal cells alive long after morphological degeneration occurred in control cultures. As shown in Fig. 7, control cultures and cultures grown in ATA appeared morphologically similar at 24 hr *in vitro* (Figs. 7A and 7B). However, by 72 hr, control cultures consisted almost entirely of pyknotic cells and cell debris (Fig. 7C), whereas in ATA, a significant fraction of input cells (20–30%) remained phase-bright and neurite-bearing (Fig. 7D). Of the cells rescued from death, nearly all were postmitotic neurons (94 ± 1.8% expressed NCAM).

Cell Types that Undergo Apoptosis in Vitro

At the time of plating, the dissociated OE neuronal cell fraction consists of ~75% NCAM$^+$ ORNs and ~25%

TABLE 1
Phenotypes of TUNEL$^+$ Cells in Olfactory Epithelium *in Vivo*

	Percentage of TUNEL$^+$ cells				
		1 day		⩾56 days	
Phenotype	0 days Unop	OBX	Cont	OBX	Cont
NCAM$^+$	86.7 ± 0.4 N = 1185	91.7 ± 3.9 N = 410	86.1 ± 0.4 N = 130	100 N = 243	93.0 ± 4.0 N = 109
Thy1.1$^+$	51.9 ± 1.0 N = 924	72.8 ± 2.0 N = 440	66.5 ± 0.9 N = 110	90.9 ± 1.0 N = 613	67.2 ± 3.6 N = 114
keratin$^+$	0 N = 865	0 N = 433	0 N = 118	0 N = 131	0 N = 141
NCAM$^-$	13.3 ± 0.4 N = 1185	8.3 ± 3.9 N = 410	13.9 ± 0.4 N = 130	0 N = 243	7.0 ± 4.0 N = 109

Note. Cryostat sections of OE from OT-2 mice that had never undergone surgery (''Unop''), or had the left olfactory bulb removed 1 or ⩾56 days prior to sacrifice, were reacted with anti-NCAM, anti-Thy1.1, or anti-keratins and then processed for TUNEL. TUNEL$^+$ cells residing within or outside layers of NCAM$^+$, Thy 1.1$^+$, or keratin$^+$ immunoreactivity were counted separately in the left (bulbectomized [''OBX'']) and right (contralateral [''Cont'']) OE of bulbectomized animals; for unoperated animals, counts from both sides were pooled. Cells lying on the borders of NCAM$^+$ or Thy 1.1$^+$ layers were counted as positive for those markers, respectively. Since Analysis of Variance (Glantz, 1992) revealed no significant differences among animals sacrificed at 8 or 12 weeks postbulbectomy, results from these two timepoints were pooled for the ⩾56-day category. Values shown are mean ± range from two animals in each category. N, total number of TUNEL$^+$ cells evaluated for each category.

NCAM$^-$ cells (presumptive INPs). During the first 24 hr in culture most INPs differentiate into postmitotic, NCAM$^+$ ORNs (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994). However, a few INPs remain, allowing us to determine whether ORN precursors, as well as postmitotic ORNs, undergo apoptosis *in vitro*. In these experiments, the TUNEL technique, NCAM-immunostaining, and [^{3}H]TdR were combined to analyze cultures fixed at 24 hr, with [^{3}H]TdR present during the final 4 hr. The results, shown in Table 3, indicate that both ORNs (37.2% of NCAM$^+$ cells) and INPs (8.7% of [^{3}H]TdR-labeled cells) undergo DNA fragmentation in cultures grown without ATA. Furthermore, ATA treatment significantly reduces DNA fragmentation in both of these cell types. Figure 8 shows an example of an S-phase neuronal precursor that is TUNEL$^+$ at this time in culture.

Effects of Neurotrophins on Cultured OE Neuronal Cells

Neurotrophins—the family of polypeptide growth factors that are structurally related to nerve growth factor (NGF)—are known to function as survival factors *in vitro* for central and peripheral neurons, including sensory neurons (e.g., Chun and Patterson, 1977; Johnson *et al.*, 1986; Davies, 1987; Henderson *et al.*, 1993; Hyman *et al.*, 1991). Neurotrophins are expressed in the olfactory bulb of the rodent brain (e.g., Large *et al.*, 1986; Guthrie and Gall, 1991; Maisonpierre *et al.*, 1990), raising the possibility that these factors exert effects on ORNs *in vivo*. To test whether ORNs are responsive to neurotrophins, we grew dissociated OE neuronal cells for 72 hr in the presence of individual neurotrophins at a variety of concentrations and then counted the total number of ORNs that were present in the cultures. In Fig. 9A, these results are expressed as percentages of the number of cells initially plated.

Treatment of OE neuronal cell cultures with three neurotrophins—BDNF, NT-3, and NT-5—significantly increased the number of phase-bright, neurite-bearing ORNs present at 72 hr, at all concentrations tested (1–100 ng/ml). NGF had no significant effect at these concentrations. Consistent with the observed positive effects of neurotrophins, the cyclic AMP analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), which mimics the survival-promoting effects of NGF on sympathetic and sensory neurons (Rydel and Greene, 1988), also caused a significant increase in the number of ORNs present at 72 hr in culture. Interestingly, no individual neurotrophin had as great an effect as ATA or CPT-cAMP (Fig. 9A).

One possible explanation for the partial effects of individual neurotrophins could be that only subpopulations of ORNs are responsive to these factors. To test this idea, we stained OE sections from neonatal mice with antibodies specific for trkA, trkB, and trkC, the receptor tyrosine kinases activated by NGF, BDNF and NT-5, and NT-3, respectively (reviewed in Chao, 1992; Clary *et al.*, 1994; Yan *et al.*, 1994; Hoehner *et al.*, 1995). The results are shown in

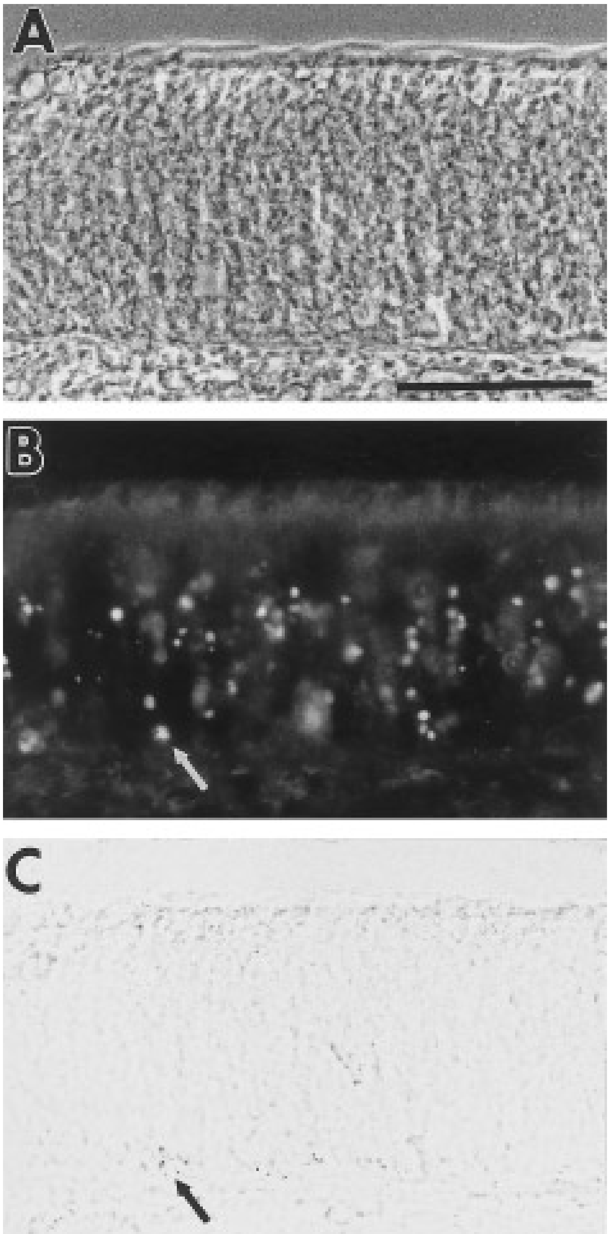

FIG. 4. ORN precursors undergo apoptotic death *in vivo*. An adult male CD-1 mouse subjected to unilateral bulbectomy was injected with [^{3}H]TdR just prior to sacrifice at 24 hr postsurgery (see Materials and Methods). OE sections were processed for TUNEL and autoradiography. Photomicrographs shown are all of the same field: (A) phase-contrast, (B) TUNEL (C) bright-field. Arrows in B and C show a cell that is TUNEL$^+$ and has incorporated [^{3}H]TdR, located in the basal compartment of the OE on the OBX side. Bar, 50 μm. Because TUNEL$^+$/[^{3}H]TdR$^+$ cells were located in the basal one-fourth of the OE, and because such cells were rare, we also processed slides from [^{3}H]TdR-injected animals for anti-keratin immunoreactivity, TUNEL, and autoradiography. Inspection of greater than 25 mm of OE from the OBX OE of such animals showed *no* TUNEL$^+$/[^{3}H]TdR$^+$ cells that were also keratin$^+$.

Figs. 9B and 9C: In neonatal OE, trkB immunoreactivity and trkC immunoreactivity are present in fractions of ORNs, throughout the epithelium. No evidence for trkA immunoreactivity could be found (data not shown).

DISCUSSION

Neuronal Cells of the OE Die with Characteristics of Apoptosis

The results show that DNA fragmentation, an established characteristic of and marker for apoptosis, occurs in both normal and target-deprived OE. The numbers and types of cells exhibiting DNA fragmentation *in vivo* indicate that apoptosis is restricted to neuronal cells (ORNs and their precursors, but not horizontal basal cells or sustentacular

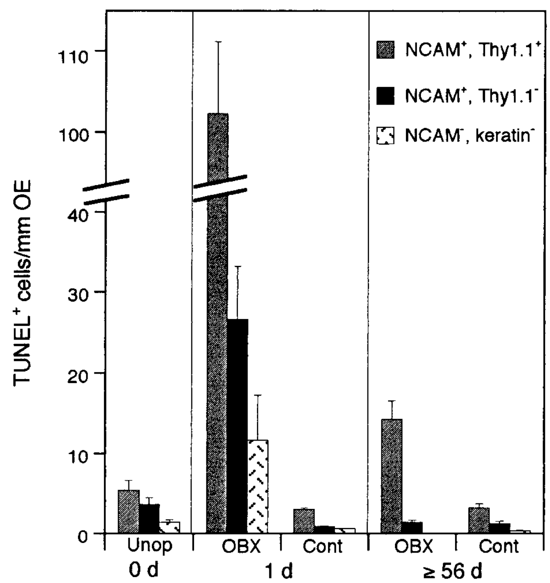

FIG. 5. Phenotypes of OE neuronal cells that undergo apoptotic death *in vivo*. This graph combines data from Fig. 2 and Table 1. Values for the NCAM$^+$/Thy1.1$^-$ category were obtained by subtraction (percentage that was Thy1.1$^+$ subtracted from percentage that was NCAM$^+$ to determine percentage that was NCAM$^+$/Thy1.1$^-$). The percentage of TUNEL$^+$ cells that were NCAM$^+$/Thy1.1$^+$, NCAM$^+$/Thy1.1$^-$, or NCAM$^-$ was multiplied by the mean number of TUNEL$^+$ cells per millimeter of OE in OT-2 mice at each timepoint [calculated from values from Figs. 2A and 2B: unoperated (10.5 $\pm$ 2.3), 1 day (140.5 $\pm$ 11.6 OBX, 4.5 $\pm$ 0.3 Cont), and $\geqslant$56 days (15.8 $\pm$ 2.6 OBX, 4.8 $\pm$ 0.7 Cont) postbulbectomy]. The derived values for TUNEL$^+$ cells/mm OE for each phenotype category are plotted, along with error bars. Errors were propagated at each stage of analysis: propagated error for differences was calculated as the root mean square of the SEMs; for products, the fractional error was calculated as the root mean square of the individual fractional errors, where fractional error = SEM/value.

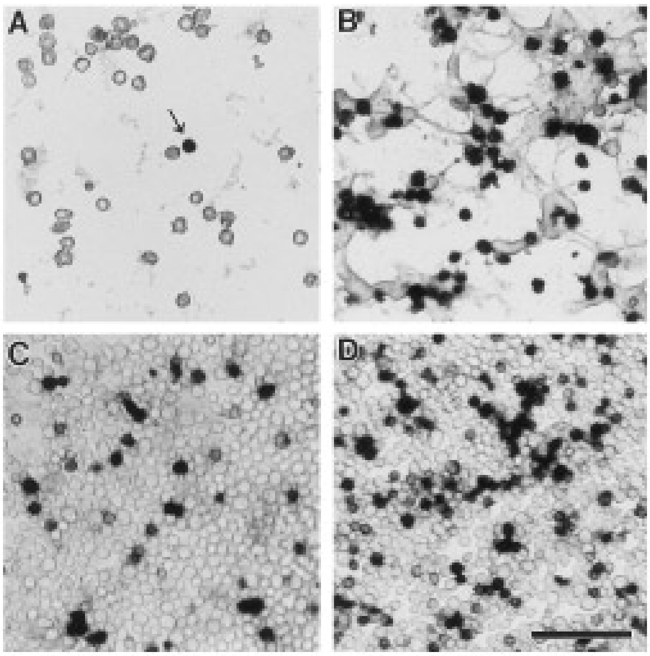

FIG. 6. DNA fragmentation in cultured olfactory neuronal cells. Dissociated OT-2 OE neuronal cells were plated at $\sim 4 \times 10^4$ cells/well in 96-well tissue culture plates, fixed after either 30 min or 24 hr of culture, and processed for TUNEL staining. Thymocyte cultures prepared from neonatal CD-1 mice were plated near confluence and cultured in the presence or absence of 1 μM dexamethasone for 4 hr before fixation and TUNEL staining. TUNEL$^+$ cells appear black in these bright-field photomicrographs. (A) Olfactory neuronal cells at $t = 30$ min, arrow points to a TUNEL$^+$ cell; (B) olfactory neuronal cells at $t = 24$ hr; (C) thymocytes, no dexamethasone; (D) dexamethasone-treated thymocytes. Bar, 50 μm.

cells); occurs constitutively (i.e., in unoperated (control) animals and on unoperated (contralateral) sides of bulbectomized animals); and increases dramatically following bulbectomy (Figs. 1 and 2).

The present study also demonstrates that cells of the OE undergo DNA fragmentation upon explantation into culture (Fig. 6), and use of the culture system permitted additional criteria to be used to verify that this DNA fragmentation is indicative of apoptosis: DNA fragmentation in OE neuronal cells could be prevented by actinomycin D, cycloheximide, and aurintricarboxylic acid (ATA) (Figs. 7 and 8; Tables 2 and 3). Actinomycin D and cycloheximide inhibit RNA and protein synthesis, respectively, actions that are consistent with a requirement for synthesis of new gene products in apoptosis (Martin *et al.*, 1988; Scott and Davies, 1990). ATA prevents apoptotic death of several types of cells, including NGF-deprived neuronally differentiated PC12 cells, by an unknown mechanism (McConkey *et al.*, 1989; Batistatou and Greene, 1991). In addition to these agents, the neurotrophins BDNF, NT3, and NT5—but not NGF—can inhibit death of ORNs to a lesser degree (Fig. 9).

All Cell Types in the ORN Lineage Can Be Induced to Undergo Apoptosis

Mature ORNs. Evidence for induced apoptosis of mature ORNs came from observations *in vivo*. Very few ORNs have matured biochemically at the developmental stage from which cultures were established in this study, so the behavior of mature ORNs could not be analyzed *in vitro*. Indeed, counts of Thy 1.1 immunoreactivity at the time of plating showed <1% of cells in cultures established from E16.5 to E17.5 OT-2 mice to be Thy 1.1$^+$ (data not shown). Mature ORNs underwent a dramatic increase in apoptotic death at 24 hr postbulbectomy (Figs. 3 and 5). Because bulbectomy both severs the axons of mature neurons and permanently removes their synaptic target tissue, the death of mature ORNs at this time could be either a consequence of injury (axotomy) or a consequence of the loss of target-derived trophic support.

It is interesting that the timecourse of death of these ORNs is conspicuously rapid compared to the responses of other neurons to axotomy. Typically, neurons in adult animals either survive axotomy or die after several days to weeks (see Snider *et al.*, 1992 for review). A substantial delay preceding neuronal death is typical even when the site of axotomy is relatively close to the neuronal soma (Berkelaar *et al.*, 1994). In contrast, the responses of mature ORNs to bulbectomy more closely resemble the responses of immature neurons in other parts of the nervous system: For example, many neuronal populations that survive axotomy relatively well in adults undergo profound and rapid death when axotomized in embryonic or neonatal animals (Snider *et al.*, 1992). This similarity between mature ORNs and ''juvenile'' neurons elsewhere is paralleled by biochemical features of ORNs: Specifically, ORNs, even when mature, retain a pattern of intermediate filament and microtubule-associated protein expression that is characteristic of immature neurons (Schwob *et al.*, 1986; Ophir and Lancet, 1988; Viereck *et al.*, 1989).

At long times following bulbectomy (8–12 weeks), a sustained elevation was observed in the number of mature ORNs undergoing apoptosis, to nearly fourfold the level on the contralateral side (Fig. 5). Others have observed a more modest sustained increase in the number of pyknotic cells in the ORN layer at long times after bulbectomy (Carr and Farbman, 1992, 1993). The larger increase seen in the present study may reflect greater sensitivity of the TUNEL technique. For example, it is reasonable to believe that dying cells become TUNEL$^+$ before they become overtly pyknotic; given that apoptotic cells are generally cleared from their local environment fairly rapidly (Kerr *et al.*, 1972), earlier detection would result in the detection of a larger number of dying cells.

In chronically bulbectomized animals, it has been reported that ~90% of ORNs survive less than 2 weeks (Schwob *et al.*, 1992), a lifespan much shorter than normal for these cells (Mackay-Sim and Kittel, 1991b). Since ORNs

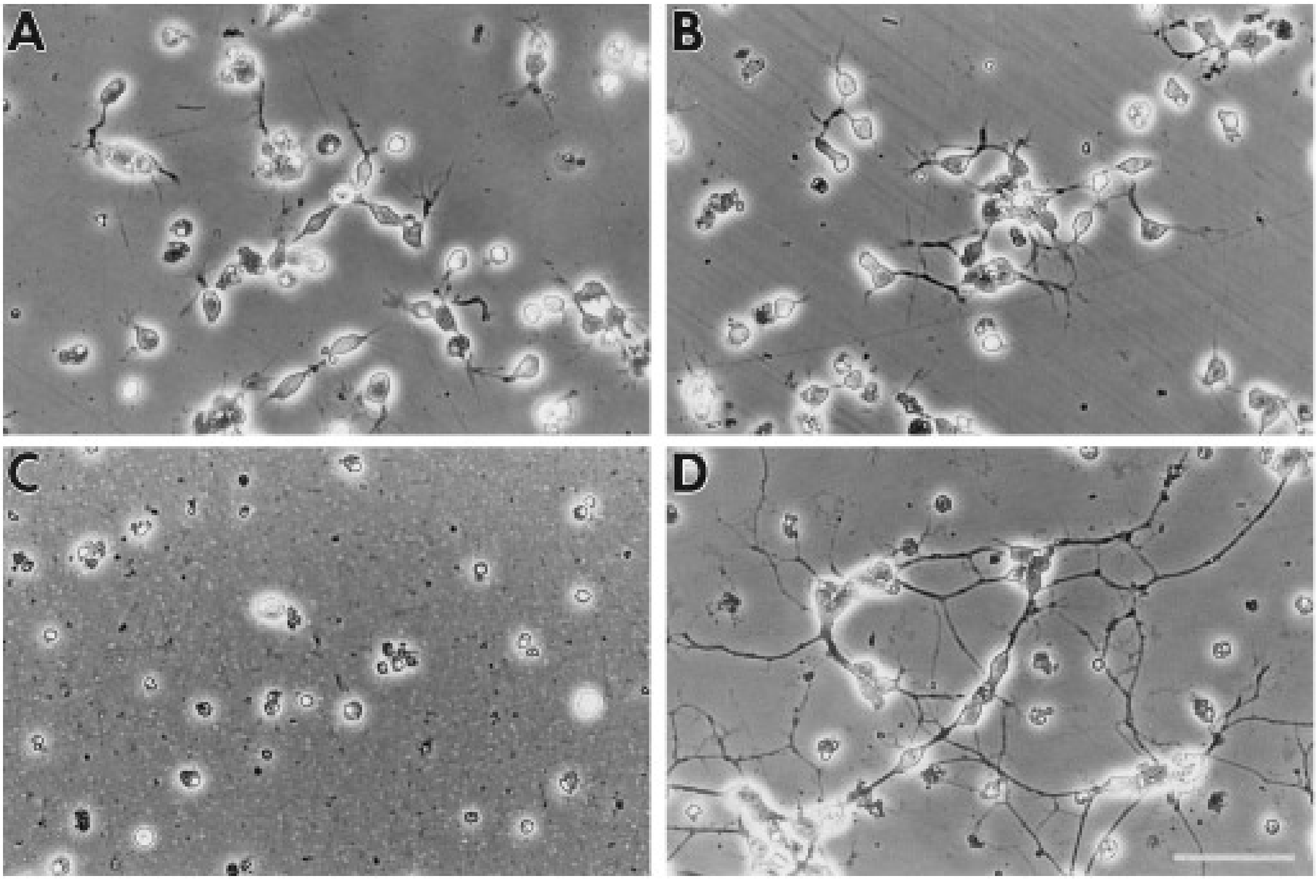

FIG. 7. Aurintricarboxylic acid (ATA) inhibits apoptotic death of olfactory neuronal cells *in vitro.* Phase-contrast micrographs of dissociated OT-2 OE neuronal cells plated at $\sim 4 \times 10^4$ cells/well in 96-well tissue culture plates with or without ATA (100 μM). (A) 24 hr *in vitro,* control. (B) 24 hr, +ATA. (C) 72 hr *in vitro,* control. (D) 72 hr, +ATA. Bar, 50 μm.

survive such a short time under these conditions, it ought to be the case that those mature ORNs that are undergoing apoptosis 8–12 weeks after bulbectomy (cf. Fig. 5) had not yet been generated at the time of surgery and therefore could not have been subjected to axotomy. Thus, the observation that mature ORN death remains elevated long after bulbectomy is consistent with the idea that death of these cells reflects not direct damage to their axons, but rather absence of their synaptic target tissue. This in turn suggests that mature ORNs depend for their continued survival on substances provided by this tissue.

Immature ORNs. Like mature ORNs, immature ORNs underwent induced apoptosis following bulbectomy. However, this effect was not sustained, and at long times following surgery, numbers of TUNEL$^+$ immature ORNs fell to baseline values (Fig. 5). These data suggest that immature ORNs are not obligatorily dependent on their synaptic target tissue for survival, while mature ORNs are (see above). This conclusion is supported by the work of others (Carr and Farbman, 1993), who have provided evidence that most ORNs that die in the chronically bulbectomized OE do so about 6–7 days after they become postmitotic, approximately the time at which ORNs become biochemically mature (Miragall and Monti Graziadei, 1982).

If immature ORNs are not dependent on the olfactory bulb for their survival, then the induced death of immature ORNs immediately following bulbectomy may be an injury response, associated either with axotomy (at least some immature ORNs should already have axons in the olfactory bulb) or with damage secondary to local changes in the OE that occur in response to the death of mature ORNs (e.g., release of substances by activated macrophages (cf. Berkelaar *et al.,* 1994)). However, it is also possible that immature ORNs are dependent for their survival on some sort of trophic support. Consistent with this latter possibility, the *in vitro* data obtained in the present study indicate that at least some immature ORNs can respond to neurotrophins (Fig. 9, and see below).

The fact that apoptotic death of immature ORNs is not increased in the chronic absence of target suggests that any

TABLE 2
Pharmacological Inhibitors of Apoptosis Inhibit DNA Fragmentation in Cultured Olfactory Neuronal Cells

Condition	% TUNEL$^+$ cells, 24 hr in culture
Control (no drug)	50.7 (±1.2)
Aurintricarboxylic acid (100 μM)	25.7 (±4.4)
Cycloheximide (6 μg/ml)	20.8 (±4.2)
Actinomycin D (6 μg/ml)	20.8 (±0.4)

Note. Dissociated CD-1 OE neuronal cells were plated at $\sim 4 \times 10^4$ cells/well in 96-well tissue culture plates with or without ATA (100 μM), cycloheximide (6 μg/ml), or actinomycin D (6 μg/ml). After 24 hr, cells were fixed and reacted for TUNEL. The number of TUNEL$^+$ cells in 10 randomly chosen fields was counted in duplicate wells for each condition; >2000 cells were counted for each value. Values shown are the mean ± range. Data are from a single typical experiment; similar effects of ATA, cycloheximide, and actinomycin D were seen in multiple independent experiments (data not shown). Assays performed separately indicated that cycloheximide and actinomycin D treatments reduced incorporation of [^{35}S]methionine into TCA-precipitable counts by 97.4 and 99.0%, respectively.

trophic support necessary for the survival of these cells is not provided by the olfactory bulb. It may be the case that such support is provided locally, e.g., by cells of the olfactory nerve or the OE itself. Thus, the data strongly imply that factors required for survival of immature and mature ORNs are likely to be different. Interestingly, the phenomenon of stage-specific switching in trophic factor dependency has been observed with other types of neurons and may be a general feature of developing neuronal systems (e.g., Buchman and Davies, 1993; Verdi and Anderson, 1994).

Immediate neuronal precursors (INPs). The finding that cells with the characteristics of INPs (NCAM$^-$, keratin$^-$ cells) also die following bulbectomy was unexpected, since these cells have no axonal processes and are located well away from the site of injury. It is possible that some of these NCAM$^-$ cells were not INPs, but rather immature ORNs that had not yet expressed NCAM (*in vitro* studies indicate a lag of ~12 hr before newly generated postmitotic ORNs express NCAM; Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994). That at least some of these NCAM$^-$, keratin$^-$ cells represented true precursors, however, was established by [^{3}H]TdR labeling, both *in vivo* and *in vitro* (Figs. 4 and 8). Although the number of [^{3}H]TdR$^+$, TUNEL$^+$ cells observed in these experiments was small, it should be pointed out that [^{3}H]TdR labeling only sets a lower limit on the actual number of INPs: Pulse-labeling with [^{3}H]TdR labeling should underestimate the number of INPs by about two- to threefold, since only INPs in S-phase will be detected (cf. DeHamer *et al.*, 1994). More importantly, if fragmentation of chromosomal DNA interferes with DNA replication—a reasonable expectation—then INPs that become TUNEL$^+$ before entering S-phase may be blocked from replicating their DNA, precluding their labeling with [^{3}H]TdR.

It is interesting to speculate how bulbectomy might induce apoptosis of INPs. Since INPs do not possess axons, they could not be directly damaged by bulbectomy. Since induced apoptosis of INPs appears to be unilateral following unilateral bulbectomy, humoral factors are not implicated in their death. Since numbers of dying INPs decrease to normal or below normal levels at long times following bulbectomy (cf. Fig. 5), it also seems unlikely that INPs are dependent for their survival on substances provided by the olfactory bulb. Instead, it seems likely that local changes in the OE, secondary to loss or removal of ORNs, trigger the death of INPs. Whether this means that ORNs provide some sort of trophic support to INPs remains to be determined. The possibility that neuronal precursors require specific survival factors has been suggested by recent studies in other systems (DiCicco-Bloom *et al.*, 1993; Birren *et al.*, 1993; Verdi and Anderson, 1994).

Neurotrophins Promote Survival of a Fraction of ORNs

In the present study, treatment of OE neuronal cell cultures with three neurotrophins—BDNF, NT-3, and NT-5—resulted in an increase in the number of phase-bright, neurite-bearing ORNs present at 72 hr in culture. It is unlikely that the observed effects of neurotrophins were secondary to effects on proliferation of ORN precursors present in the starting OE neuronal cell preparation, because the neurotrophins NGF, NT3, and BDNF have all been tested and found to have no effect on proliferation of ORN precursors from embryonic mouse OE (DeHamer *et al.*, 1994). In addition, the number of neuronal precursors ([^{3}H]TdR$^+$ incorporating cells) present in these cultures is relatively small to begin with, and nearly all of these differentiate quickly into neurons—even in the absence of any exogenous factors—and thereby disappear within 24 hr of culture (Calof and Chikaraishi, 1989; DeHamer *et al.*, 1994; cf. Table 3). Thus, the simplest interpretation of these results is that neurotrophins promote survival of postmitotic ORNs.

The finding that BDNF, NT-3, and NT-5 all support ORN survival is consistent with our observation of trkB and trkC immunoreactivity in OE of neonatal mice (Figs. 9B and 9C). The antibodies used for these experiments included ones specific for forms of trkB and trkC that contain cytoplasmic tyrosine kinase domains (Middlemas *et al.*, 1991; Lamballe *et al.*, 1991; Deckner *et al.*, 1993; Hoehner *et al.*, 1995). Thus, our results indicate that trk receptor isoforms that are capable of binding BDNF, NT3, and NT5, and eliciting intracellular signals (Berkemeier *et al.*, 1991; Klein *et al.*, 1991; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et*

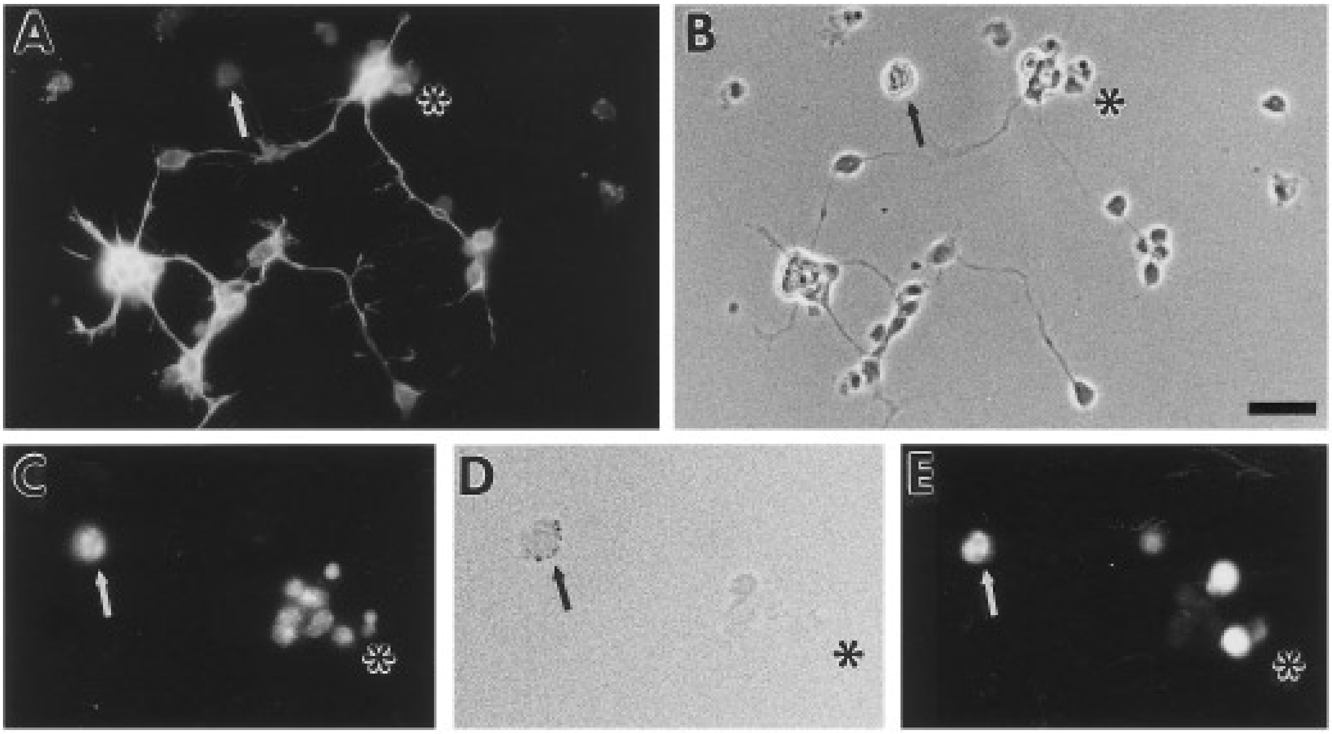

FIG. 8. Apoptotic death of ORN precursors *in vitro.* Dissociated OT-2 OE neuronal cells were plated at $\sim 10^5$ cells per glass coverslip ($\pm$ATA, 100 μM). Cells were labeled with [^{3}H]TdR (2.5 μCi per ml) from 20–24 hr in culture, fixed, stained for TUNEL and NCAM immunoreactivity, and processed for autoradiography. (A) NCAM immunostaining; (B) phase contrast of same field of cells as A showing a TUNEL$^+$ precursor, enlarged in (C) Hoechst, (D) bright-field, and (E) TUNEL. Arrows in A–E show the same NCAM$^-$, [^{3}H]TdR$^+$, TUNEL$^+$ precursor cell. To provide a point of reference, the asterisk in each panel marks the cluster of cells in the upper part of A and B. Bar, 20 μm.

al., 1991), appear to be present in OE. No effect of NGF on ORN survival was observed in these studies, consistent with observations of others on postnatal rat ORNs (Mahanthappa and Schwarting, 1993). In addition, we found no immunohistochemical evidence for expression of the NGF receptor trkA in OE (using the monospecific antibody anti-rtrkA.EX2 (Clary *et al.*, 1994; Verdi and Anderson, 1994); data not shown).

Although statistically significant in every case, the survival-promoting effects of BDNF, NT-3, and NT-5 on ORNs were never as great as that of ATA or another agent that promoted survival, CPT-cAMP (Fig. 9A). One possible explanation for the partial effects of individual neurotrophins could be that different subpopulations of ORNs are dependent for their survival on these neurotrophins. Our finding that trkB and trkC expression in the OE appear to be limited to fractions of ORNs, at least in neonatal OE, is consistent with this idea (Figs. 9B and 9C). Indeed, in other types of sensory neurons (e.g., of the dorsal root ganglia), data from neurotrophin gene ‘‘knockout’’ experiments demonstrate that different subpopulations of sensory neurons are dependent for their survival on BDNF versus NT3 (Fariñas *et al.,* 1994; Jones *et al.,* 1994).

Another possible explanation for the partial effects of these three neurotrophins has to do with heterogeneity in the state of maturity of the ORNs being cultured. Virtually all of the cells that survive in these cultures at 72 hr are *immature* postmitotic ORNs, i.e., although all express NCAM, when cultured from OT-2 embryos, they do not express Thy1.1, a marker for mature ORNs (data not shown). Thus, the relatively small survival-promoting effect of neurotrophins on embryonic ORNs may reflect the fact that only a small subset of these neurons expresses neurotrophin receptors at the developmental stages tested here. Consistent with this idea, work by others suggests that a much greater percentage of ORNs expresses trkB in adult rat OE (Deckner *et al.*, 1993), suggesting that ORNs cultured from adult OE—were it possible to prepare such cultures—might survive in greater numbers in response to BDNF and NT-5.

Finally, and perhaps most likely, the observation of partial effects of neurotrophins on cell survival may be due to

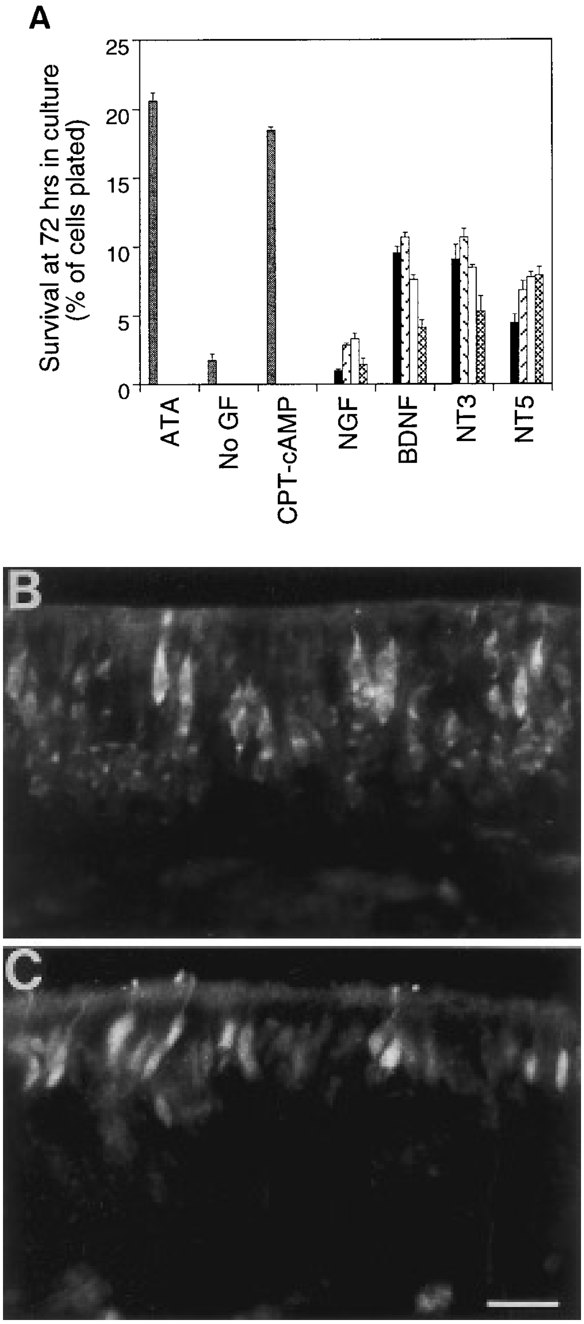

FIG. 9. Neurotrophins promote survival of cultured ORNs. (A) Dissociated OT-2 OE neuronal cells were plated at ~1.5×10^4 cells/well in 96-well tissue culture plates. Cells were plated with or without ATA (100 μM), 8-(4-chlorophenylthio)-cAMP (1 μM), or individual neurotrophins at 100 ng/ml (black bars), 33 ng/ml (lightly stippled bars), 10 ng/ml (white bars), or 1 ng/ml (darkly stippled bars) added. At 48 hr in culture, half of the medium was removed from each well and replaced with new medium containing the appropriate factor. The percentage of initially plated cells that remained viable (phase-bright and neurite-bearing) was counted for triplicate wells in each condition after 72 hr in culture. Mean percentage survival ± SEM is plotted for each condition. Compared to control, NGF did not show a significant effect at any concentration, while BDNF, NT3, and NT5 promoted survival ($P < 0.05$) at all four concentrations tested (ANOVA followed by Dunnett's test for multiple comparisons against a single control; Glantz, 1992). (B) Paraffin section of OE from a P1 OT-2 mouse stained with anti-trkB (794). Similar results were obtained using anti-trkB$_{23-36}$ (data not shown). (C) Cryostat section of OE from a PO OT-2 mouse stained with anti-trkCin2. The photomicrographs show immunoreactivity to both antibodies in scattered ORNs. Absorption of anti-trkCin2 with an excess of peptide immunogen (MILVDGQPRQAK-GELGL, 200 μg/ml) completely abolished staining. Bar, 20 μm.

TABLE 3

Cell Types Undergoing Apoptosis *in Vitro*

Cell type	Control medium	ATA (100 μM)
% of total cells that are TUNEL$^+$	44.2 ± 1.3	31.0 ± 0.83*
% of [^{3}H]TdR$^+$ cells that are TUNEL$^+$	8.7 ± 0.52	3.57 ± 0.93*
% of NCAM$^+$ cells that are TUNEL$^+$	37.2 ± 1.05	23.8 ± 1.20*

Note. Dissociated OT-2 OE neuronal cells were plated at ~1×10^5 cells per glass coverslip, with or without ATA (100 μM, labeled with [^{3}H]TdR$^+$ from 20 to 24 hr in culture, then fixed and processed for TUNEL, NCAM immunoreactivity, and autoradiography. Three coverslips were analyzed for each condition, with >600 cells counted per coverslip. To assess the fraction of [^{3}H]TdR$^+$ cells that was TUNEL$^+$, *all*[^{3}H]TdR$^+$ cells were counted on each of three coverslips in each condition (~150 [^{3}H]TdR$^+$ cells per coverslip). Data shown are from a single typical experiment; ATA treatment resulted in similar percentage decreases in TUNEL$^+$ cells of all categories (total cells, [^{3}H]TdR$^+$ cells, and NCAM$^+$ cells) in independent repetitions of this experiment (data not shown). Data are given as the mean ± SEM.

* Significantly different from control, $P < 0.05$, Student's *t* test; Glantz, 1992.

the fact that other factors, in addition to neurotrophins, are necessary for survival of ORNs. In the case of spinal motor neurons, for example, both the three neurotrophins found to be effective in the present study, and glial cell line-derived neurotrophic factor, have been found to promote survival *in vitro* (Henderson *et al.*, 1993; Oppenheim *et al.*, 1995). In this regard, it is interesting to note that, in a study using cultures of postnatal rat ORNs, Mahanthappa and Schwarting (1993) were able to detect effects of BDNF on ORN viability, but only if TGFβ2 was added to the cultures as well.

Neuronal Birth and Death in the OE

Proliferation of neuronal precursors in the OE increases following bulbectomy, reaching a peak 5–6 days postsurgery in the mouse (Schwartz-Levy *et al.*, 1991). The level of [^{3}H]TdR incorporation then declines rapidly, but remains elevated above control levels as long as 7 weeks postsurgery (Carr and Farbman, 1992). One possible explanation for this phenomenon is that the loss of ORNs following bulbectomy provides the signal for neuronal precursor proliferation. For example, differentiated ORNs might normally provide negative feedback that inhibits precursor proliferation. Similar regulatory mechanisms have been suggested for larval frog retina (Reh and Tully, 1986). Alternatively, actively dying ORNs could provide a positive signal that stimulates neuronal precursor proliferation.

It is interesting to evaluate these alternative hypotheses in view of the results of the present study. As shown in Fig. 2C, as well as in the studies of others (Costanzo and Graziadei, 1983; Schwartz-Levey *et al.*, 1991), changes in the number of ORNs in the bulbectomized OE (as reflected in epithelial thickness, which reaches its lowest point 5–6 days postsurgery and then increases to ~70% of its original value) closely parallel the changes that occur in overall [^{3}H]-TdR incorporation (Schwartz-Levy *et al.*, 1991). In contrast, numbers of apoptotic ORNs rise following bulbectomy, then fall to above-normal levels, but not in synchrony with changes in neuronal precursor proliferation. Specifically, the peak of ORN apoptosis occurs at 48 hr (Fig. 2C), 3–4 days before the peak in [^{3}H]TdR incorporation.

Recent evidence indicates that multiple rounds of division and multiple cell stages separate the neuronal stem cell of the OE from postmitotic ORNs: *In vivo* and *in vitro* studies indicate that INPs are transit-amplifying cells that divide two or more times before giving rise to neurons (MacKay-Sim and Kittel, 1991a; DeHamer *et al.*, 1994). Additional data suggest that INPs are themselves the product of yet another early progenitor, also not a stem cell (Gordon *et al.*, 1995). Consequently, if dying ORNs provide a positive signal for proliferation, then that signal is likely to be specific for an early cell in the ORN lineage (perhaps the stem cell), since geometric expansion of that cell's progeny would account for the delayed peak in overall [^{3}H]TdR incorporation. By similar reasoning, if living ORNs provide a negative signal for proliferation, then that signal is probably *not* directed at an early cell in the ORN lineage (at least not exclusively). Thus, the question of the cellular stage(s) at which neuronal precursor proliferation is regulated in the OE, and the question of whether neurons that are living or ones that are dying are responsible for regulating proliferation, are closely linked.

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