

Naturally Occurring Cell Death during Postnatal Development of the Substantia Nigra Pars Compacta of Rat

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We have quantified degenerating cells during the postnatal development of the substantia nigra pars compacta (SNpc) of normal rat. The cells are identified at the light microscope level by the characteristic morphologic appearance of large, rounded clumps of chromatin, stained intensely with both Nissl and silver stains. These cells were most numerous on Postnatal Day (PND) 2 and rapidly declined in number by PND 12. However, on PND 14, there was a second peak in the number of pyknotic cells. Their number again rapidly diminished to very few by PND 28. Many of these cells appear neuronal in origin. We conclude that naturally occurring cell death is a prominent feature of normal nigra development, and we hypothesize that the magnitude of this regressive event may be regulated by target (striatal)-derived trophic support. © 1993 Academic Press, Inc.

INTRODUCTION

Apoptosis refers to a characteristic morphologic pattern of cell death in which there is breakdown of nuclear chromatin into discrete clumps, associated with initial preservation of intracellular organelles (1). This morphologic pattern is frequently observed during developmental cell death in the central nervous system (2) and other tissues. While the phenomenon of developmental cell death (also termed programmed or naturally occurring cell death) in the nervous system has been recognized for many years, it is only since the landmark studies of Hamburger and Levi-Montalcini in the 1940s (3) that the phenomenon has gained widespread recognition as an important and common developmental process (2, 4, 5). Interest in this process has also been generated by many observations that it is regulated by hormones (6), trophic factors (7), and other factors. In addition, it has been shown in several paradigms that, rather than being a passive event, developmental cell death requires active gene expression (8-10).

Our interest in naturally occurring cell death arises from the observation that developmental axon-sparing

striatal injury in postnatal rat results in a decreased adult number of dopaminergic neurons in the substantia nigra (SN) (11). This decrease in neurons occurs following either a hypoxic-ischemic or excitotoxic (quinolinic acid) lesion of the target striatum in the absence of direct injury to the nigra. In these injuries, dopaminergic terminals within the striatum are spared by many morphologic and biochemical criteria (12-14). One possible explanation for the decrease in SN neurons is that a reduction in the striatal target site has led to an augmented developmental cell death event in the developing SN, resulting in a diminished adult number of neurons. Such a sequence of events has been demonstrated to occur for several neuronal populations in chick, including the lumbar motor neurons, ciliary ganglion, and isthmooptic nucleus (reviewed in (4)), and for trochlear motor neurons in duck (15). In these paradigms, augmented cell death occurs in the setting of ongoing, normal developmental death. If such a mechanism applies to SN development, then we would anticipate that normal developmental death would likewise occur in nigra. While developmental cell death has been previously shown to be widespread in the postnatal rat brain (16), there has not been a demonstration of its occurrence or time course in SN. We have, therefore, analyzed this phenomenon in SN in the postnatal rat, using morphologic criteria to identify and quantitate degenerating cells.

METHODS

Female rats 12-14 days pregnant were obtained from Charles River Laboratories (Wilmington, MA). Each rat was monitored for delivery in the afternoon of each day, and the day of delivery was defined as Postnatal Day (PND 1). Starting with PND 2, groups of 4 or 5 pups were sacrificed every other postnatal day until PND 16. A total of 35 animals among 11 litters was used. In order to distribute any variability due to litter differences, each developmental time point from PND 2 to PND 16 was represented by pups from 2 or 3 litters. Pups were unsexed, and both males and females were used. Three additional groups were sacrificed on PND 20, 28, and 70.

On the day of sacrifice, pups were anesthetized with Metofane by inhalation. Adults (PND 70) were anesthetized with pentobarbital 60 mg/kg ip. Each rat was perfused intracardially with 0.9% saline for 1 min by gravity, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.1) for 20 min at room temperature. The brain was then removed and postfixed in the same fixative for at least 4 weeks for the immature brains and 2 weeks for the adult brains. The day before sectioning, each brain was placed in 20% sucrose in fixative. On the day of sectioning, each brain was rapidly frozen by immersion in isopentane on dry ice and then brought to -21°C in a cryostat. Serial coronal sections through the SN were cut at 30 μm . Alternating sections were used for silver and Nissl staining. Sections for silver staining were collected free-floating in cold fixative. Sections for Nissl staining were thaw-mounted onto gelatin-subbed slides.

Serial sections were Nissl stained using thionin. Free-floating sections were silver stained using the method described by Nadler and Evenson (17). Briefly, all incubations were done in acid-washed, covered glassware at room temperature. Sections were carried through solutions free-floating in custom-made plastic grids with nylon mesh bottoms. Sections were first washed in distilled water and were then incubated in a pretreating solution (equal volumes of 9% NaOH and 1.2% NH_4NO_3). They were then placed in impregnating solution (containing 0.25% AgNO_3) for 10 min, followed by a wash, and then development. Sections were then mounted on acid-washed slides, subbed in 0.05% chromium potassium sulfate/0.5% gelatin. After drying, sections were washed again in 0.5% acetic acid, followed by water, dehydrated through alcohols, cleared in xylenes, and coverslipped.

In order to quantitate the number of dying cells in precisely the same region at different times of development, we counted cells in the substantia nigra pars compacta (SNpc) only in sections that contained the medial terminal nucleus accessory optic tract (MT). In our previous studies in adult animals (11), we found that this structure is restricted in its rostrocaudal extent and its presence identifies a narrow, well-delineated plane. In addition, it is clearly identifiable at PND 1 (Fig. 113 in Ref. (18)) and in adulthood (Plane 3.7, Paxinos-Watson (19)). To count the number of dying cells, we scanned all available Nissl sections containing the MT tract (2-4 sections for PND 2 and 4; 3-6 for all other ages) at 400 \times . We expressed counts as degenerating cells per MT plane. The SNpc was scanned on one side only. At early postnatal times, the SNpc does not have distinct boundaries (see Fig. 113 in Ref. (18)). Thus, at these early time points we can only say with confidence that we were scanning the region of the SNpc. This region was defined as that dorsal to the SN pars reticulata (which was encompassed on its ventral surface by the cerebral peduncle) and extended from the lateral border of the MT tract to the lateral extent of the cerebral peduncle, i.e., the regions defined as SN compacta

and lateralis of Fig. 113 in Ref. (18). At 400 \times , pyknotic cells were identified by the presence of one or more round, dark-staining masses of chromatin surrounded by cytoplasm. In the absence of cytoplasm, single circular dark-staining masses were not counted. There were two reasons for this policy. The first is that a single pyknotic cell can give rise to multiple dark staining masses (see Figs. 1 and 2); consequently, counting of these masses in the absence of surrounding cytoplasm could lead to an overestimation of the number of degenerating cells. Second, at late postnatal ages, occasional single dark-staining nuclear masses are observed in normal brain tissues that do not have a counterpart on silver staining. The nature and cellular origin of this staining are thus unclear, so we avoided counting such profiles. Counts were performed independently by the two investigators and, in the event of discrepancies, sections were reviewed and a final count was agreed upon. Since there did not appear to be a change in the average size of pyknotic cells with development, we did not perform an Abercrombie correction (20) on data from different developmental time points; results are expressed as uncorrected counts.

The numbers of cells per SNpc MT plane were averaged to determine a mean for each animal. At each time point, the means for the animals (4 or 5 per time point) were averaged to give a mean number of dying cells per SNpc MT plane at that time.

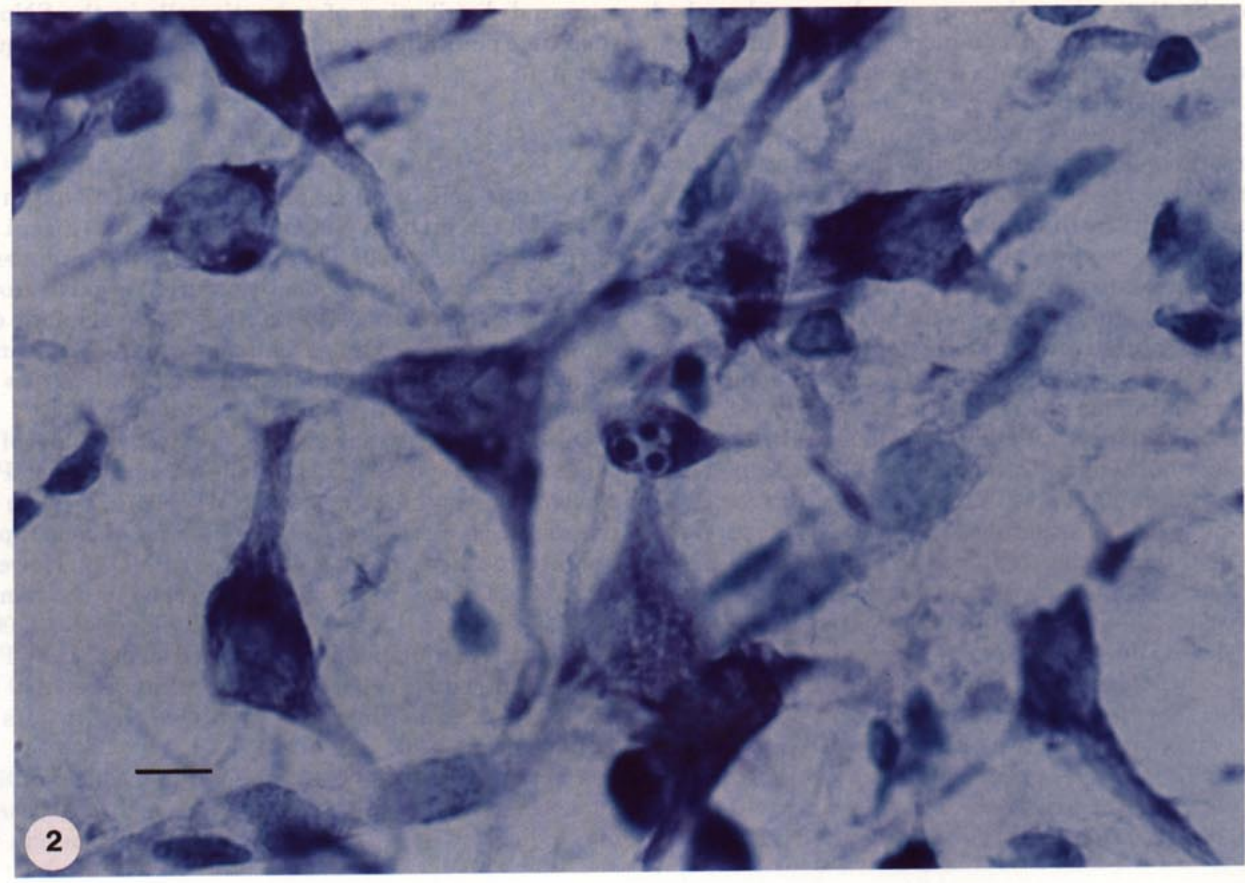
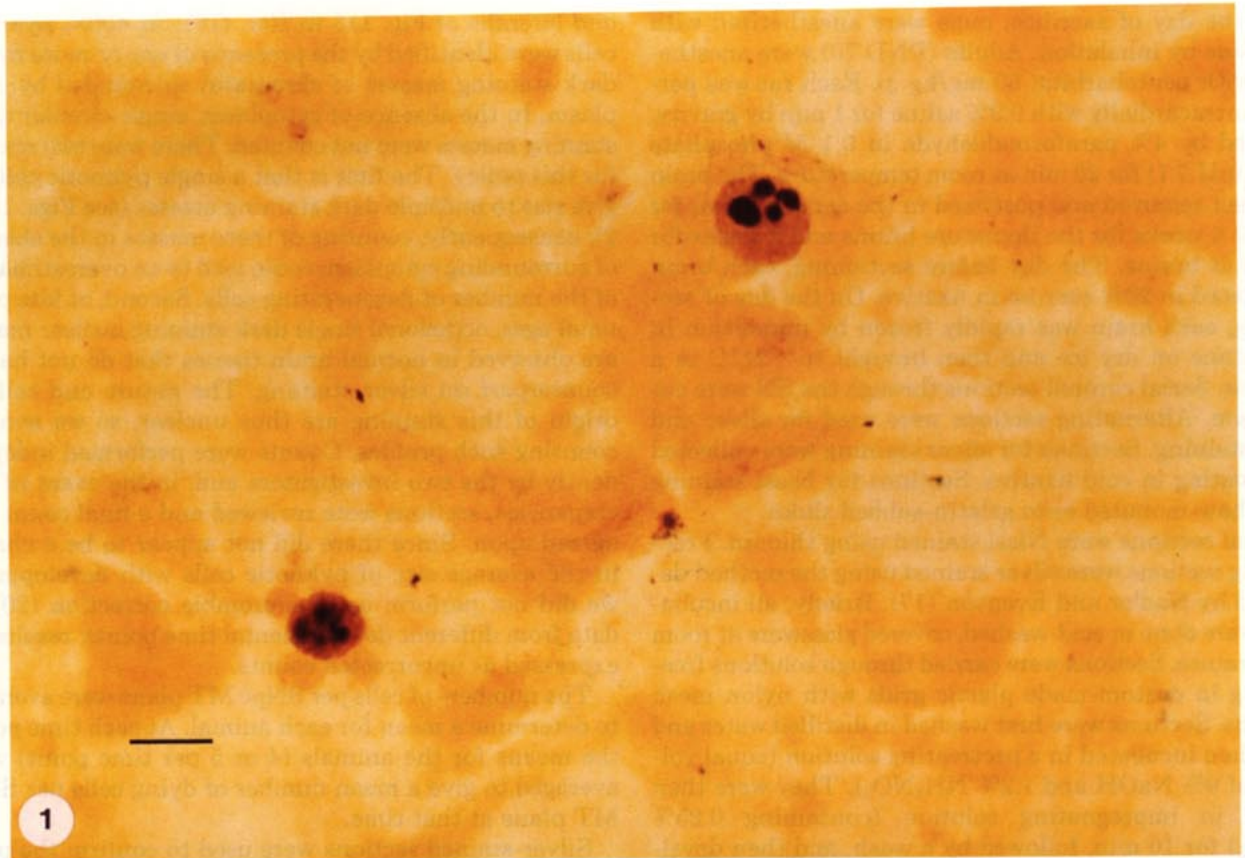
Silver-stained sections were used to confirm the presence and distribution of pyknotic cells in the SNpc at each time point; they were not used for purposes of quantification.

RESULTS

Pyknotic cells were readily observed in SNpc in the postnatal rat, on both Nissl and silver stains (Figs. 1 and 2) from PND 2 to 20. These cells were most numerous on PND 2, when they frequently occurred in small groups. At early postnatal times, as on PND 2, the pyknotic cells showed multiple, rounded clumps of chromatin and a rounded surrounding cytoplasm. In general, it was not possible to identify the cell type of origin.

On quantitative analysis, the greatest number of degenerating cells per SNpc MT plane was observed on PND 2, when a mean of 9.6 ± 1.2 (SEM) cells/MT plane was counted (Fig. 3). Thereafter, the number of cells rapidly declined to a nadir of 1.2 ± 0.2 cells by PND 12. However, on PND 14, there was a clear resurgence in the number of cells observed, 4.5 ± 0.3 cells/MT plane ($P = 0.0001$, ANOVA). The number again declined such that by PND 28 only rare single cells were observed in occasional sections. No degenerating cells were observed in rats examined at PND 70.

At later postnatal stages, many dying cells identified by chromatin clumping retained enough cytoplasmic morphology to suggest that the cell type of origin was



neuronal, as shown in Fig. 2. This cell retains an angulated appearance at one pole, with an emanating process that appears dendritic.

In general, throughout postnatal development, pyknotic cells appeared singly or in small groups throughout the extent of the SNpc; there was no clear clustering in either medial or lateral regions or within the dorsal and ventral tiers of the SNpc. This was also true during the apparent resurgence of cell death on PND 14; cells were observed singly in medial, central, and lateral regions of the SNpc.

DISCUSSION

We have demonstrated that developmental cell death occurs postnatally in the SNpc, as it does in many other regions of the rat central nervous system (16). The morphology of the dying cells observed on both Nissl and silver stains is typical of that described for apoptosis (1), with the formation of round, darkly staining clumps of chromatin. Ultimately, examination at the ultrastructural level will be required to confirm the preservation of intracellular organelles during the initial phase of cell death, another morphologic hallmark of apoptosis (1). At later postnatal time points, many cells retained morphologic characteristics, indicating that they were neuronal in origin.

In this study, we have not counted the number of living Nissl-stained neurons at different developmental time points; therefore, we do not know, in absolute terms, the magnitude of the neuronal death that takes place postnatally. In other systems, peripheral and central, it ranges from 16 to 84%, depending on species, structure, and the developmental period examined (4, 21, 22). The difficulty in quantifying the absolute number of neurons in the SNpc throughout development lies in the fact that, unlike other nuclei and regions previously studied (4, 21, 22), it does not have distinct boundaries throughout development. It is likely, however, that the cell death we have observed leads to a reduction in the number of neurons in the SNpc between birth and adulthood. In rat, the genesis of SN neurons, studied with [³H]thymidine, occurs between Embryonic Days 12–15 (23, 24). In addition, nigral precursor neurons have completed their migration from the subventricular zone to the SN by Embryonic Day 18 (25, 26). Thus, by birth, it is likely that the SNpc has obtained its full complement of neurons and, therefore, postnatal cell death would result in a decrement in that number. While the number of dying cells counted

per section is small, their number probably underestimates the actual number of cells that die, because dying cells appear to be present only briefly *in vivo* (27).

The biphasic occurrence of cell death in the SNpc, with a peak at PND 2 and a second at PND 14, was not expected. In most prior studies of developmental cell death, there appears to be only a single major time period of cell loss (for example, see Refs. (21, 22, 28)). However, it is possible that counting the number of living cells at different developmental time points is not sufficiently sensitive to detect substantial differences in the rate of cell death; such differences would appear only as changes in the slope of living cell number plotted as a function of time. Quantitation of the number of dying cells, as performed by Gould *et al.* (22), may be more sensitive to changes in rates of cell death. In a study of naturally occurring cell death in rat cortex, Ferrer and co-workers quantified the number of dying cells, as we have done, and noted a second peak of cell death at PND 13 in the subcortical plate (29). It must be noted that our conclusion that a resurgence of cell death occurs at PND 14 rests on the assumption that the duration of time that a dying cell takes up the silver and Nissl stains is brief and constant throughout development. Only under such conditions will the number of dying cells accurately reflect the *incidence* of cell death. If the duration of stainability is prolonged, then counts of stained cells would reflect the *prevalence* of cell death; the prevalence, in turn, could be increased by a longer duration in stainability, without an actual change in incidence. However, in most systems examined, dying cells exist for only brief periods of time (10, 27).

The cause of the resurgence in cell death at PND 14 is unknown. In most neuronal groups showing developmental cell death, the regressive event occurs during, or just after, the time that the group of cells begins to establish connections with its projection field (30). In the rat, the period of maximal synaptogenesis in the striatum, studied by quantitative ultrastructural study of synapse formation, occurs between Postnatal Days 13 and 17 (31). Thus, the peak of cell death at PND 14 may relate to competition among SNpc neurons for synaptic contact within striatum. If such a mechanism applies to the peak of cell death at PND 14, then possibly a distinct mechanism applies to the death observed earlier in the postnatal period. At that time, there are very few synapses formed in the striatum (31), and there are low levels of striatal markers of dopaminergic terminals (32). The cells dying in the earlier postnatal period may be a different population from those

FIG. 1. Silver stain of SNpc at PND 2. Two pyknotic cells are identified by multiple, darkly staining chromatin clumps surrounded by a rounded cytoplasm that stains slightly more than that of normal neurons, which appear golden yellow. The cell to the left is partially out of the plane of focus. Bar = 10 μ m.

FIG. 2. Nissl stain of SNpc at PND 8. A single pyknotic cell with three darkly staining chromatin clumps is observed in the center of the field. While the cytoplasm of this cell is shrunken, it retains an angulated appearance on the right side, with an emanating process that appears to be a dendrite. Bar = 10 μ m.

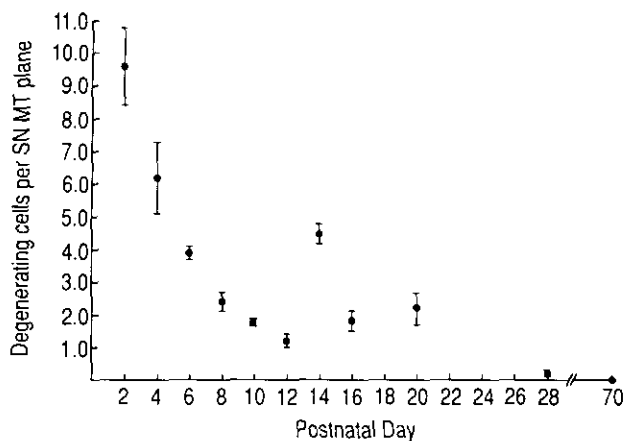


FIG. 3. The postnatal time course of developmental cell death in SNpc. At each postnatal day indicated, the total number of pyknotic cells in SNpc in sections containing the medial terminal nucleus accessory optic tract (MT) were counted on Nissl-stained material. Two to six sections per animal were counted, on one side, and averaged to give a mean count for that animal. For each time point, the mean counts for all animals ($N = 4-5$ per time point) were averaged. Bars represent standard error of the mean. Pyknotic cells were most numerous at PND 2, and they then rapidly declined in number. However, a resurgence in cell death occurred on PND 14 ($P = 0.0001$, ANOVA on PND 8 to 28; $P < 0.01$, PND 14 versus all other groups, Newman-Keuls post-hoc analysis). By PND 28, cell death had virtually ceased.

dying at PND 14; they may be nonneuronal, or, if neuronal, they may be nondopaminergic cells. Alternatively, these may be dopaminergic neurons, but they may be dying for reasons unrelated to their terminal projections; there may be death mechanisms related to their afferent projections or to systemic factors, such as hormones or locally produced trophic factors.

For methodological reasons, we have restricted this analysis to a single plane of the SNpc to achieve consistency in the region examined at different developmental time points. Therefore, we do not know if there are rostrocaudal gradients in the cell death process in SNpc. We did examine for the presence of different times of cell death in the ventral and dorsal tiers of the SNpc. It is known that the ventral tier neurons (which project to striatal striosomes) develop earlier than those in the dorsal tier (which project to the striatal matrix) (33). However, we did not at any time postnatally observe clustering of dying cells in either tier; specifically, we did not observe clustering of dying cells in the ventral tier on PND 2 or in the dorsal tier on PND 14.

Our observation that developmental cell death occurs in the SNpc makes plausible our hypothesis that a decrease in the size of the striatum during development, due to an axon-sparing injury, results in a diminished adult number of SNpc dopaminergic neurons because diminished striatal trophic support (provided in retrograde or anterograde fashion) results in an augmentation of the normal cell death event. This hypothesis must be examined further by quantitation of cell death in SNpc following developmental striatal injury.

The present demonstration of cell death in SNpc may have implications for human disease. Since there is evidence that naturally occurring cell death is an active event,

mediated by gene expression (8-10), it is possible that abnormalities in the regulation or expression of these genes may play a role in pathologic degeneration of SNpc cells in adult life in diseases such as Parkinson's disease. While environmental factors have been thought to probably play a major role in this disease (34), there is some evidence that genetic factors may play a role as well (35).

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