

Research report

# The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra

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## Abstract

We have previously shown that apoptotic natural cell death occurs within the substantia nigra (SN) pars compacta of the rat postnatally. However, the occurrence of natural cell death in phenotypically defined dopaminergic neurons has not previously been identified, nor has its time course been defined in pre- or postnatal development. We therefore examined the SN at intervals from E19 to P28 using immunostaining for tyrosine hydroxylase with a Nissl counterstain to identify intranuclear apoptotic chromatin clumps. We have found that natural cell death in dopaminergic neurons is biphasic. An initial, broad peak begins at E20, reaches maximum at P2, and abates by P8. A second peak occurs at P14. We conclude that most of the natural cell death in this neuronal population occurs in the early postnatal period.

*Keywords:* Natural cell death; Apoptosis; Substantia nigra; Dopaminergic; Developmental; Parkinson's disease

## 1. Introduction

Many developing neural systems undergo a period of natural cell death during development, which is regulated by target-derived trophic factors, and which determines the number of neurons which survive into maturity [6]. Regulation of the mature number of dopaminergic neurons of the substantia nigra (SN) is of particular interest, because it is these neurons which degenerate in human Parkinson's disease, and regulation of their number may influence susceptibility to this condition. We have previously shown that natural cell death, with the morphology of apoptosis defined by Nissl or silver stain, occurs postnatally within the cytoarchitectonic boundaries of the SN pars compacta (SNpc) [10]. This natural cell death event is inducible by either early target injury [14] or early destruction of dopaminergic terminals [16]. Early target injury leads to a persistent reduction in the mature number of dopaminergic neurons [4]. In our earlier study of natural cell death in SNpc, we observed two apparent peaks in the prevalence of apoptotic profiles; one at postnatal day (P)2, and a second at P14 [10]. We did not, however, ascertain whether cell death began prior to P2, or whether the apparent

biphasic time course of cell death could be attributed to differences in cell phenotype. To address these issues, we have here identified dopaminergic neurons by tyrosine hydroxylase (TH) immunostaining, and apoptotic nuclear chromatin clumps by Nissl counterstain. We have previously shown that profiles identified by discrete, rounded chromatin clumps within the SNpc are confirmed to be apoptotic by both ultrastructural analysis and in situ end labeling [14].

## 2. Materials and methods

### 2.1. Animals

Timed-pregnant rats were obtained from Charles River (Wilmington, MA). The vaginal plug date was defined as embryonic day (E)1, and the day of birth was defined as P1. A total of 65 animals from 13 litters was used.

### 2.2. Histology

For the prenatal studies, the dam was anesthetized with pentobarbital (45 mg/kg), pups were removed individually by Caesarean section and anesthetized by hypothermia. Mean crown-rump length at embryonic ages were: E19 =

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23 mm; E20 = 30 mm; E21 = 41 mm. Each pup was perfused through the left ventricle with 0.9% saline at 4°C for 1 min followed by 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.1) for 5 min. The brain was post-fixed in situ within the skull in the same fixative for 36 h, then removed and post-fixed for an additional 2.5 weeks. Postnatal pups were anesthetized with Metofane by inhalation, and processed in similar fashion, except the duration of perfusion was 10 min. Each brain was cryoprotected in 20% sucrose in fixative, rapidly frozen in 2-methylbutane on dry ice and cut in a cryostat at 20  $\mu\text{m}$ . Sections were alternately thaw-mounted on slides for a strict set of Nissl-stained serial sections, or free-floated for TH immunostaining.

### 2.3. TH immunostaining

TH immunostaining was performed with a monoclonal antibody to TH (Boehringer-Mannheim) at 1:10, utilizing a biotinylated horse anti-mouse secondary antibody (Vector) and the avidin-biotin technique (ABC, Vector), as previously described [17]. TH-immunostained sections were then Nissl-counterstained to identify chromatin clumps.

### 2.4. Quantitative morphology

The cross-sectional anatomy of the SN varies according to its rostro-caudal location. Therefore, for quantitative morphologic analysis, the SN was divided into a caudal, central and anterior subdivision at each developmental age. For E19 brains, the caudal subdivision was defined by a compact cluster of TH-positive neurons in the ventro-lateral midbrain bounded dorso-medially by the medial lemniscus, and ventrally by the cerebral peduncle (see Fig. 59 of Paxinos et al. [19]). The central subdivision was defined by the presence of the fasciculus retroflexus abutting the ventral surface of the brain (see Fig. 57, Paxinos et al. [19]). The anterior subdivision was defined as that portion of SN anterior to the point where the fasciculus retroflexus separated from the ventral surface (see Fig. 55). The E20 and E21 brains were divided in similar fashion, except the medial terminal nucleus of the accessory optic tract (MT) was used to define the central subdivision, and the anterior subdivision was defined as all SN sections anterior to MT. Postnatal brains were similarly divided except that the caudal-most boundary was defined by the first appearance of the SNpc with a clear medial to lateral linear appearance. For the embryonic brains,  $\geq 2$  TH-stained SN sections per subdivision were analyzed ( $11.7 \pm 0.3$  SN sections per brain). For the postnatal brains, 4 SN sections from each subdivision were analyzed. Apoptotic profile counts from sections within a subdivision were averaged to give a profile count for that subdivision. In order to determine the total number of apoptotic profiles for each subdivision, the total number of sections within that subdivision was counted on the strict serial Nissl-stained set,

and that number was multiplied by the mean number of profiles/section [11]. The total number of profiles for the SN was then calculated as the sum of the total number for each SN subdivision.

The volume of the SN was determined as the sum of the volume of the three subdivisions. The volume of each subdivision was determined as the product of its length and its mean cross-sectional area as determined on representative TH-stained sections using a Loats Associates Inquiry image analysis system.

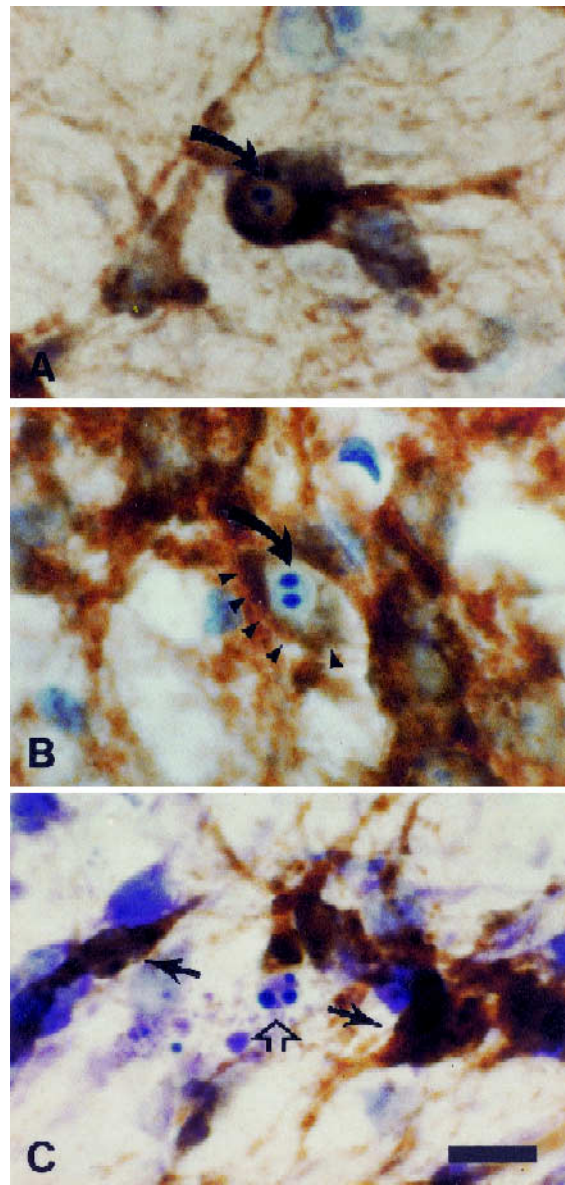


Fig. 1. Apoptotic profiles in SNpc. A: This is an example of basophilic apoptotic chromatin clumps (curved arrow) within a neuron in the SNpc immunostained for TH at P2. B: a second example of basophilic apoptotic chromatin clumps (curved arrow) within a TH-positive neuron. The edge of the neuron is marked by arrowheads. This cell was located within the SNpc of a P4 rat. C: an apoptotic profile within SNpc by regional criteria. The profile (clear arrow) contains 3 basophilic chromatin clumps and is within 15  $\mu\text{m}$  of two TH-positive neurons indicated by solid arrows. Bar = 10  $\mu\text{m}$ .

The number of apoptotic profiles in each section was quantified by scanning the entire SN at  $600\times$  to identify characteristic basophilic, rounded, intranuclear chromatin clumps. Each was counted according to whether it fulfilled cellular criteria for being TH-positive or regional criteria for being within the TH-positive neuron group (i.e., SNpc-positive). To fulfill cellular criteria, an apoptotic nuclear profile was required to be encompassed by TH-positive cytoplasm within the same plane of focus (Fig. 1A,B). To fulfill regional criteria, an apoptotic nuclear profile was required to be within  $15\ \mu\text{m}$  of two TH-positive neurons (Fig. 1C). Counts of apoptotic profiles obtained using the two sets of criteria were highly correlated ( $r = 0.939$ ,  $P < 0.0001$ ). Apoptotic profiles were counted only if their tops were within the plane of the section, based on the stereologic principle of the optical disector

[8]. We observed that apoptotic profiles in a section were not observed on strictly adjacent sections, and thus were not split by the microtome. A similar observation has been made by Clarke and Oppenheim [5]. Based on our three-dimensional assessment, and on the absence of split profiles, we did not use traditional corrections for double-counting.

### 3. Results

At E19, negligible numbers of apoptotic profiles were observed (Fig. 2B,C). Apoptotic profiles first consistently appeared in sections on E20, and then rapidly increased in their prevalence to reach a maximum by P2. Data derived from cellular and regional criteria followed virtually identical temporal patterns. By P8–12 apoptotic profile levels

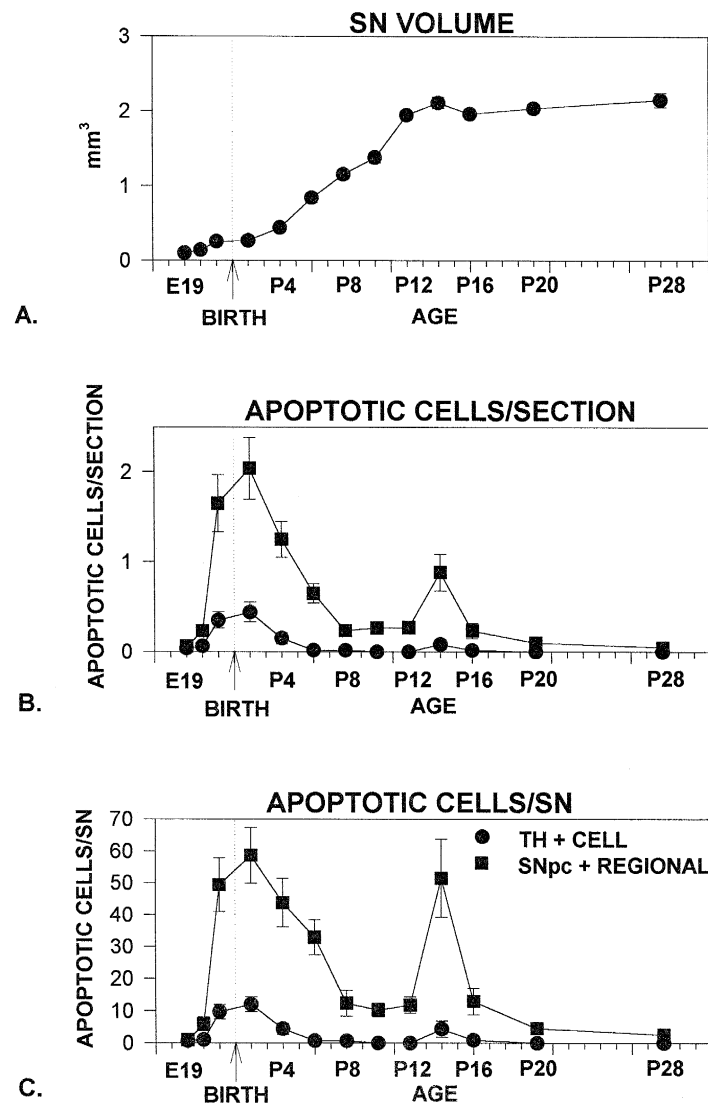


Fig. 2. Time course of natural cell death in dopaminergic neurons of the SN. A: this curve indicates the growth of the volume of the SN from E19 to P28. Note there is an inflection point at P14. B: this curve shows the mean number of apoptotic profiles per section, meeting cellular or regional criteria, during development. C: this curve shows the mean number of profiles per SN during development. The curves in B and C show a major early peak in natural cell death from E20 to P8 and a second, minor peak at P14.

reached stable, low levels, but on P14 there was a recrudescence in the number of profiles, as quantified by both cellular and regional criteria. This second peak in prevalence was present in data expressed either as number of profiles/section (Fig. 2B) or as number of profiles per SN (Fig. 2C), and corresponds to our previous observation of a second peak in apoptotic profiles counted in SNpc on Nissl-stained sections [10]. Interestingly, at the time of this second peak, there was an inflection in the total volume of the SN (Fig. 2A).

We considered the possibility that the apparent biphasic nature of the time course of developmental cell death among defined dopaminergic neurons could be due to separate epochs of cell death in separate locations within the SN. We therefore analyzed the prevalence of apoptotic profiles, positive by regional criteria, according to their location in the caudal, central and anterior subdivisions of SN, defined for each developmental age (Fig. 3). We found that in all 3 subdivisions the time course of cell death was biphasic. In addition, while we did not perform a quantitative analysis, we did not readily observe a medial-to-lateral difference in the timing of cell death that would account for the biphasic time course.

There was no apparent difference in the morphology of apoptotic profiles observed in the early period of cell death, as compared to the later period at P14. Nor was there a difference by quantitative analysis of mean cross-sectional area. Nissl-stained profiles which were among the TH-positive neurons by regional criteria at P2 had a mean area of  $33.2 \mu\text{m}^2$  ( $n = 40$ ); those at P14 were not significantly different, with a mean area of  $30.4 \mu\text{m}^2$  ( $n = 51$ ).

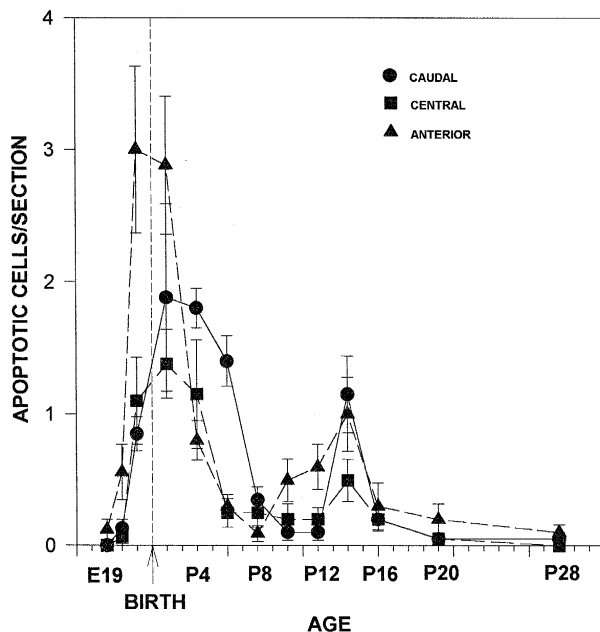


Fig. 3. Time course of natural cell death in dopaminergic neurons of the SN, analyzed according to rostro-caudal location. The time course of cell death is similar in the three subregions.

#### 4. Discussion

These results show that apoptotic nuclear chromatin clumps can be identified by Nissl stain within TH-positive neurons of the SNpc during normal development in the rat. These results extend our previous observation that apoptotic profiles can be identified within SNpc both by Nissl and silver staining [10] by showing that these profiles can be identified within dopaminergic neurons. The conclusion that these chromatin clumps indicate apoptotic cell death is supported by our prior demonstrations that such basophilic clumps occur in apoptotic cells in the SN identified by ultrastructural [14] and in situ end-labeling criteria [14,17]. Other investigators have successfully used the characteristic appearance of basophilic chromatin clumps to identify apoptosis in the central nervous system [5]. While the demonstration of apoptotic profiles in defined dopaminergic neurons makes clear that natural cell death occurs in this population, it is notable that these profiles are few in number. Even at the peak of natural cell death, there were fewer than 1 per section. There are several possible reasons for their small number. Apoptosis is a rapid process, estimated to occur over a few hours [18], and thus few apoptotic cells may be identifiable at a given time in spite of massive cell death. In addition, only a fraction of the apoptotic profiles within the SNpc were located within TH-positive neurons. It is possible that many of these profiles derived from dopaminergic neurons, and yet could not be identified within a TH-positive cytoplasm. This inability to demonstrate TH-positive cytoplasm could be explained on the basis of several factors: (1) loss of expression of TH due to the cell death process; (2) physical separation of the nucleus from the cytoplasm as the cell undergoes apoptosis; and (3) limited penetration of immunoreagents, resulting in false negatives for TH immunoreactivity. Based on these considerations, we chose to quantify apoptotic profiles in proximity to TH-positive neurons as well as those within them. It is likely that many apoptotic profiles positive by regional criteria derive from dopaminergic neurons. This conclusion is based on the high correlation between counts based on cellular and regional criteria, and the exact temporal correspondence between them during the course of development.

Our quantitative analysis indicates that a major natural cell death event takes place in defined dopaminergic neurons between E20 and P8. This finding is consistent with the observations of Tepper and colleagues showing that there is a significant reduction in the number of TH-positive neurons in the SN between P1 and P7 [25]. In our study, we did not examine whether natural cell death occurs in dopaminergic neurons prior to E19. These tissues are more difficult to process free-floating, and therefore will require a different methodological approach. TH immunostaining is first identifiable in midbrain at E12.5 [23]. Dopaminergic neurons of the SN undergo mitosis from E12 to E15 [12,15], and they migrate from the subventricu-

lar zone to the ventral mesencephalon until E18 [22]. Therefore, it would not be possible to equate the presence of natural cell death among dopaminergic neurons at these early embryonic ages with a lasting reduction in the ultimate adult number; dying neurons may be replaced with newly formed or inwardly migrating neurons. However, natural cell death in the period we have studied, which is after the completion of mitosis and migration into the SN, does diminish the ultimate number of these neurons surviving into adulthood.

Our analysis also indicates that a second peak of natural cell death occurs within dopaminergic neurons at P14. Thus, our original observation of a biphasic time course within SNpc [10] has been replicated within this neuronal phenotype. This second peak is not accounted for by different timing of natural cell death within different subregions in the rostro-caudal dimension. The basis of this biphasic time course is unknown, but its existence suggests that the mechanisms of regulation may differ between the two phases. Often, the period of target contact and competition for synapse formation corresponds with the period of maximal natural cell death [18]. The greatest increase in the number of synapses in the developing striatum takes place between P13 and 17 [9]. It is therefore possible that the peak of cell death at P14 corresponds to a period of competition for target support mediated via synaptic contact. Even if this were so, however, it would remain to be determined what factors are the principal regulators of the earlier, larger cell death event. Although these two phases of cell death may differ in their regulatory control, they are identical in the morphologic features of apoptotic profiles.

Although our prior observations that either lesion of the striatal target [14] or of dopaminergic terminals [16] results in induced cell death in dopaminergic neurons would suggest that these neurons are target-dependent during development, it is unknown what striatal factors might support their viability. It has been suggested that GDNF may be a trophic factor for the support of dopamine neurons, based on its ability to support them in embryonic culture [13], its mRNA expression in striatum during development [2,20,21,24], its retrograde transport by dopaminergic neurons [27], and its ability to support the viability of dopamine neurons in injury models [1,3,7,26]. However, it is not yet known whether GDNF or any other factor attenuates natural cell death in dopamine neurons.

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