Published in final edited form as:

Exp Neurol. 2007 April ; 204(2): 802–807. doi:10.1016/j.expneurol.2007.01.009.

Apoptotic natural cell death in developing primate dopamine midbrain neurons occurs during a restricted period in the second trimester of gestation

Bret A. Morrow^a, Robert H. Roth^{a,b}, D. Eugene Redmond Jr^{a,c}, John R. Sladek Jr^d, and John D. Elsworth^{a,*}

^aDepartment of Psychiatry, Yale University School of Medicine, 300 George St, 8300 Suite, New Haven, CT 06511, USA

^bDepartment of Pharmacology, Yale University School of Medicine, 300 George St, 8300 Suite, New Haven, CT 06511, USA

^cDepartment of Neurosurgery, Yale University School of Medicine, 300 George St, 8300 Suite, New Haven, CT 06511, USA

^dDepartment of Psychiatry, University of Colorado Health Sciences Center, Denver, CO 80262, USA

Abstract

Natural cell death (NCD) by apoptosis is a normal developmental event in most neuronal populations, and is a determinant of the eventual size of a population. We decided to examine the timing and extent of NCD of the midbrain dopamine system in a primate species, as dopamine deficiency or excess has been implicated in several disorders. Genetic or environmental differences may alter the extent of NCD and predispose individuals to neurological or psychiatric diseases. In developing rats, NCD in the midbrain dopamine system has been observed to start at the end of gestation and peak in the postnatal period. In fetal monkey brains, apoptosis in midbrain DA neurons was identified histologically by chromatin clumping in tyrosine hydroxylase-positive cells, and confirmed by TUNEL and active caspase-3 staining. A distinct peak of NCD occurred at about E80, midway through gestation in this species. We estimate that at least 50% of the population may be lost in this process. In other brains we determined biochemically that the onset of apoptosis coincides with the time of greatest rate of increase of striatal DA concentration. Thus, marked apoptotic NCD occurs in the primate midbrain dopamine system half-way through gestation, and appears to be associated with the rapid developmental increase in striatal dopamine innervation.

Keywords

dopamine; substantia nigra; apoptosis; development; Parkinson's disease; striatum; tyrosine hydroxylase; primate; monkey

© 2007 Elsevier Inc. All rights reserved.

*Corresponding author: John D Elsworth PhD, Departments of Psychiatry, Yale University School of Medicine, 300 George St, 8300 Suite, New Haven, CT 06511, USA, john.elsworth@yale.edu, t: (203) 785-4506, f: (203) 785-5275.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Natural cell death (NCD) of a large percentage of a neuronal population, primarily through apoptosis, is a normal developmental occurrence (Clarke, 1999, Oppenheim, 1991). Such loss of cells occurs relatively late in maturation, typically following expression of phenotype and projection to target regions. Regression of a neuronal population could serve several beneficial functions, such as elimination of aberrant connections or adjustment of a neuronal population that was initially greater than required for the necessary connections and signaling purposes of the system (Kuan et al., 2000, Oppenheim, 1991).

Abnormalities in the development of dopamine neurons have been hypothesized to be involved in the etiology of several disorders, such as Parkinson's disease (Carvey et al., 2006, Ramsden et al., 2001), Lesch-Nyhan disease (LND) (Wong et al., 1996), and schizophrenia (Rehn Rees, 2005, Weinberger, 1987). As NCD is an important determinant of the size and function of a neuronal population, it is possible that genetically- or environmentally-induced differences in the extent of NCD of DA neurons could predispose individuals to such neurological or psychiatric diseases

In order to understand intrinsic and extrinsic factors that may alter the extent of NCD in DA neurons in man, it is important to determine the timing and magnitude of this event in a primate species. In the rodent, NCD in midbrain DA neurons occurs postnatally (Oo Burke, 1997), but this may not be the case in primates, bearing in mind rodent-primate differences in development (Bayer et al., 1993). Thus, we examined histologically a series of normal fetal and neonatal monkey brains for signs of apoptosis in midbrain DA neurons, and compared frequency of apoptosis with maturation of the nigrostriatal DA system.

Material and Methods

Animals and tissue collection

The gestational age of fetal African green (vervet) monkeys (*Chlorocebus aethiops sabaues*) collected from the St. Kitts Biomedical Research Foundation was estimated by ultrasound and/or direct measurement of fetal femur length, as described in Morrow et al. (2005). These studies were approved by our institutional animal use and care committee (IACUC). Brains for immunohistochemistry (n=13) were fixed by cardiac perfusion with heparin-containing saline followed by 100-500 ml 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3). The ages ranged from embryonic day (E) 70-167, and the mean gestation in this species is 165 days. Each brain was post-fixed in cold paraformaldehyde solution for 24-72 hours, before transfer to cold 30% sucrose in phosphate buffer. The midbrain region was cut into 40 µm sections and stored as 5-10 sets of tissue each having sections 200-400 µm apart. A single set of tissues then represents a uniform sampling of the midbrain starting at a random point anterior to the A9/A10 region. Brains of older fetuses were cut into a larger number of sets. Fetal brains for monoamine measurements (n=10) were not perfused, being dissected in cold Ringer's solution before freezing in liquid nitrogen. Neonatal brains (n=5) and young adult brains (n=11) were perfused with saline, blocked and dissected on a refrigerated surface, with remaining tissue being post-fixed, as described before. The mean ages of the neonates was postnatal day (P)19, and range was between P11-25.

Estimation of apoptosis using chromatin clumping

A free-floating set of tissue from each fetal brain was immunostained for TH-ir using published methods (Morrow et al., 2002, Morrow et al., 2005). Briefly, TH-ir was identified using a primary antibody (1:1000 overnight at room temperature, MAB-318, Chemicon, Temecula, CA) with the ABC technique using a Vectastain Elite kit (Vector Labs, Burlingame, CA) and visualized with a diaminobenzidine (DAB) reaction (0.05% DAB and 0.002% hydrogen peroxide) (Hsu et al., 1981). Sections were mounted onto glass slides and

lightly counterstained for Nissl substance using thionin to visualize nuclei and nucleoli. Apoptotic profiles were counted only if they fulfilled the criteria of having, in the same focal plane, both chromatin clumps within the nucleus together with TH-ir in the cytoplasm (Marti et al., 1997).

TH-ir, presumed dopaminergic, cells in A9 and A10 regions of fetal primate midbrains were quantified using a modified single section disector method (Howard Reed, 1998, Moller et al., 1990) on an immunostained set of serial sections using a microscope (BH-2, 50x oil, Olympus, Melville, NY) with a computer-controlled stage (Optiscan, Prior Scientific, Rockland, MA) as we have previously reported (Morrow et al., 2005). Briefly, an unbiased counting frame (97×97 µm) and a systematic, random sampling technique was used to estimate the number of TH-ir cells, and the number of TH-ir cells with apoptotic changes. The counting frame was randomly placed at a starting point outside the region of interest on each section and systematically moved following a “grid” pattern across the entire region of interest on each slide. DA neurons were defined by the appearance of Nissl stained nucleoli within the nuclear region of TH-ir cells inside the unbiased counting frame. Apoptotic cells were estimated by counting appearance of groupings of chromatin clumps either in TH-ir cells or surrounded by TH-ir material within the counting frame. The volume of the A9/A10 was determined using the Cavalieri grid technique (Howard Reed, 1998). The estimated total numbers of TH-ir cells, and apoptotic TH-ir cells were calculated by multiplying the respective counts by the ratio of the total A9/A10 area to the area sampled in the count frame.

Verification of apoptotic profiles using TUNEL or caspase-3

Apoptosis was recognized in midbrain cells by DNA fragmentation, detected by the TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL) method. The free-floating sections from 3 fetal monkeys were used, 2 (E79 & E81) from within the apoptotic window identified the appearance of chromatin clumping, and 1 outside this developmental window (E120). Tissue sections were permeabilized by treatments with either 0.5% Triton X-100, or 0.3% triton X-100 followed by 0.3 µg/ml proteinase K (Bessert Skoff, 1999) before using an assay kit (ApopTag, catalog # S7101, Chemicon). In one case (E79), the sections were subsequently double-labeled by processing them for TH-ir (as above). Although double-staining for TUNEL and TH-ir was successful, the quality of immunostaining with this procedure was not as good as that obtained in tissue stained just for TH-ir.

The appearance of the cleaved form of caspase-3, another marker of apoptosis, was examined in a separate set of midbrain tissue sections from 3 fetal monkeys (E79, E81 and E167). Fixed brain sections were incubated with anti-caspase antibody (anti-ACTIVE caspase-3, catalog number G7481, 1:125; Promega Corp., Madison, WI) overnight, followed by a biotinylated anti-rabbit IgG and vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions using nickel-intensified DAB (0.04% DAB, 2.5% nickel sulfate and 0.005% hydrogen peroxide) to visualize targets as a black stain. DA neurons were visualized in the caspase-3-ir sections using anti-TH antibody, as previously described above, to yield a red-brown product.

Determination of dopamine tissue levels

DA and metabolites were determined using HPLC with electrochemical detection, using a slight modification of our previous method (Elsworth et al., 1989). Tissue samples were sonicated in cold 0.1 M perchloric acid, containing 0.13 mM EDTA and dihydroxybenzylamine as internal standard using a Branson Cell Disrupter on setting 3 for a few one-second bursts. The homogenate was centrifuged (45,000 g, 4 °C, 20 min.), and the

pellet saved for protein determination (Pierce Biotechnology Inc., Rockford, IL) after hydrolyzing with 1M NaOH. A portion of the supernatant was mixed with 3M Tris base to raise the pH to about 8.2 and immediately transferred to a small alumina column, in order to isolate selectively and concentrate the catechols. The column was centrifuged briefly (100 g, 2 min), washed with water and re-centrifuged. DA and other catechols were eluted from the alumina column with 0.1M oxalic acid. An aliquot was separated on a small bore (100 × 4.6 mm) reverse-phase (3 micron, C18) HPLC column ("Microsorb", Varian Inc., Palo Alto, CA). The ratio of the peak heights for DA and dihydroxybenzylamine was determined for each sample and the concentration of DA present was calculated by referring to standards. The mobile phase, pumped at 0.4 mls/min, contained sodium citrate (30 mM), sodium phosphate (14 mM), sodium octanesulphonate (2.3 mM), EDTA (0.025 mM), acetonitrile (6.5%), tetrahydrofuran (0.6%) and diethylamine (0.1%) at pH 3.2. The glassy-carbon amperometric detector was maintained at a working electrode potential of 0.70 volts vs Ag/AgCl reference electrode (BAS). A detection limit of 5-10 fmol DA was achieved. The remaining portion of the supernatant was filtered (0.2 micron) used for direct injection (no alumina separation) on the HPLC column for separation of 5HT, 5HIAA and HVA, as described above, except that the flow rate was increased to 0.8 ml/min.

Results

Chromatin clumping in TH+ cells of A9 and A10 regions was observed in all examined specimens between E70 and E100 (Fig. 1A and 1B). Outside this window, only one TH-ir cell in each of two samples was observed with chromatin clumping. Fig. 2 shows that the frequency of chromatin clumping in midbrain TH-ir cells peaked at about E80, and peak rate of observed positive cells was about 5% of TH-ir neurons present at that time.

As a confirmation of apoptosis, sections from the midbrain of a smaller number of brains were processed for TUNEL staining. Frequent positive staining was only observed in tissue from specimens taken within the peak of apoptosis as defined by chromatin clumping. TUNEL staining appeared either as a nuclear restricted reaction, as apparent cellular debris (Fig. 1D), or as diffuse staining not contained in any identified cellular compartment (Fig. 1E and F). We also observed examples of chromatin clumping within a TUNEL stained body (Fig. 1D) or cells displaying both TUNEL and TH-ir, although both these occurrences were fairly rare. Although chromatin clumping was our quantitative measure of apoptosis in DA neurons, we did estimate the frequency of TUNEL staining in the analyzed samples. However, the tissue processing made the sections more fragile than normal and a few were lost, making the estimate semi-quantitative. However, when expressed as number of TUNEL-positive nuclei per unilateral substantia nigra tissue section, the 2 samples taken at about E80 had 14 and 9 cells, whereas the sample taken later in gestation had less than 1.

As a secondary marker of apoptosis, selected fetal midbrains were tested for immunoreactivity to activated caspase-3. As shown in Fig. 1C, positive staining was seen in the samples at E79 and E81, but not in the E167 sample. Immunoreactivity to activated caspase-3 occurred in the cytoplasm and processes of most cells that were labeled. This distribution of labeling has been seen before (Jeon et al., 1999).

The monoamine determinations (Fig. 3) showed that striatal DA levels were low, but detectable between E34 and E70, after which time striatal concentrations rose sharply. Between E86 and birth, DA levels in the striatum climbed more steadily. The mean striatal level of DA midway through the first month of life was approximately two-thirds the adult concentration.

Discussion

The present data indicate that a period of natural cell death for midbrain dopamine neurons in non-human primates peaks at mid-gestation (E80). In the rat, natural death in midbrain DA neurons has been identified during the late prenatal and early postnatal period, with the rate maximal at P2 (Oo Burke, 1997). While the mid-gestational peak of this event in the monkey contrasts with its postnatal occurrence in the rat, it is important to note that there is a poor relationship between the timing of birth and the maturational state of the brain in different species. However, there is a strong conservation in the sequence of neural events across mammalian species. Using the data provided by Clancy et al. (2001), it is striking that the developmental time of P2 in the rat is equivalent to E78 in the rhesus monkey, a species with the same gestational period as the vervet monkey. Thus it would appear that natural cell death of midbrain dopamine neurons occurs at the same developmental stage in rodents and primates, although the timing with respect to gestation and birth is very different.

In the rat a second, smaller peak of NCD in midbrain DA neurons occurs at about P14 (Oo Burke, 1997), an age in the rat that is estimated to be equivalent to E127 in the monkey (Clancy et al., 2001). We observed no evidence of cell death in our samples between E120 and E130. We also examined a series of neonatal monkey brains that covered the same early neonatal period (P11-P25) in which NCD was seen in rat DA neurons and did not observe any TH-ir neurons with signs of apoptosis. Thus, it appears that the second wave of apoptosis in rat DA neurons does not have a counterpart in the monkey.

In order to understand the significance of the NCD for midbrain DA neurons, it is relevant to ask what developmental events coincide with these periods. Oo and Burke (1997) speculated that the later wave of NCD in the rat may be related to the period of target contact and competition for synapse formation, as the rate of synaptogenesis is greatest rate between P13-17 (Hattori McGeer, 1973). In the rhesus monkey, synaptogenesis in the striatum begins at E65, has the greatest rate of increase at about the time of birth, and synaptic density achieves a plateau at about P30 (Brand Rakic, 1984). However, it is also important to refer to estimates of the time at which terminals of developing DA neurons reach the striatum, as the data for striatal synapse formation is not specific to dopaminergic contacts (Goldman-Rakic, 1981). In the vervet monkey we have observed that TH-ir fibers first impinge upon the posterior striatum between E55-60, and continue to increase in density until after birth (Bundock, 1999, Sladek et al., 1995). As shown in Fig. 3, the greatest rate of increase in striatal DA concentration occurs between E70-90, the time when our data indicates that apoptosis is peaking in substantia nigra DA neurons. During this time, striatal DA concentration rises from 3% to 35% of adult levels. The actual trigger of apoptosis cannot be discerned from this study. However as striatal DA levels are a good marker of dopaminergic terminal density (Onn et al., 1986, Wilson et al., 1996), our data suggest that apoptosis in this population coincides with the time at which the dopaminergic innervation of the striatum is increasing at its greatest rate. The establishment of the next 35% of adult striatal DA levels (35-70%), which occurs more slowly, is not associated with any detectable natural cell death in this population. This phase of maturation may reflect an increase in DA production in each neuron rather than an increase in the number of terminals (Morrow et al., 2005).

In addition to determining when natural cell death occurs in primate midbrain DA neurons, another goal of the present study was to estimate the extent of apoptosis that occurs during this time. The peak rate of apoptosis was 5% of the TH+ neurons, at about E80. It should be noted that this percentage is based on the number of cells TH+ at the time of examination, rather than as a percentage of the eventual number of cells expressing detectable TH immunoreactivity. If the latter denominator is used the rate of apoptosis at E80 is 2%. This

estimate is higher than in the rat, where the highest incidence of apoptosis was observed to be 10 TH+ cells per substantia nigra and 60 apoptotic profiles per substantia nigra (Oo Burke, 1997). This apparent difference could be due either to a real difference in the proportion of developing DA neurons that undergo apoptosis or to a difference in the duration of the apoptotic process in rodents and primates. The peak rate of apoptosis (5%) in our study is somewhat higher than that found in the one study using human fetal brain (periaqueductal gray, 2.85%), although the samples in this latter study (Chan Yew, 1998) were spaced several weeks apart, so a definite peak could not be identified. Over the 25 day span in which we detected apoptosis in TH+ cells, the mean rate was 2.6%/day. However, it is not possible to translate reliably this number into the proportion of the population that is sacrificed by NCD. This is because of the time a cell can retain the characteristics of apoptosis is not known. Generally the apoptotic process is thought to be a rather rapid event, taking a only few hours to complete (Bursch et al., 1990, Oppenheim, 1991). However, NCD of central neurons in vivo may be more protracted. In fact, it has been shown in the rat that the elimination of the apoptotic granule hippocampus cells induced by adrenalectomy took 72 hours (Hu et al., 1997). The end stages of cell disassembly would not meet our criteria for inclusion, but based on Hu et al. (1997) it is possible that our study included cells in which apoptosis started 24-48 hours earlier. Using this figure, we estimate that at least 50% of the final population was removed during this window of NCD.

While it appears that the timing of apoptotic cell death in primate nigrostriatal DA neurons is associated with the initial innervation of the striatum and formation of synapses, the actual biochemical triggers for the process are not known. However, recent investigations of NCD in DA neurons of mice strongly implicate a role for GDNF as a critical target-derived factor in regulating the magnitude of NCD. Thus, intrastriatal injection of anti-GDNF neutralizing antibodies were found to augment the extent of NCD, while over-expression of GDNF led to an increased number of DA neurons after the first phase of NCD (Kholodilov et al., 2004, Oo et al., 2003).

The extent of NCD in nigrostriatal DA neurons conceivably may have an impact on later susceptibility to disorders such as Parkinson's disease. As the degree of NCD is a determinant of an individual's endowment of nigrostriatal DA neurons and as these neurons appear to be progressively lost during aging (Fearnley Lees, 1991, Forno, 1996), it is possible that an unusually large degree of NCD may increase the risk of acquiring Parkinson's disease later in life. It is also possible that while undergoing NCD, neurons may be more susceptible to insults than at other times, and our preliminary data appear to support this (Elsworth et al., 2005). Previous investigations have revealed that the period of most rapid brain growth ("the brain growth spurt") is a time when rats respond to NMDA antagonists and GABA-A agonists with apoptotic neurodegeneration in many brain regions (Ikonomidou et al., 2001). In the rat the brain growth spurt peaks at approximately 7 days after birth, for rhesus monkeys the peak growth takes place after approximately 115 days of gestation, and in man this occurs during the first month of life (Dobbing Sands, 1979). It is quite possible that the brain growth spurt and the time of NCD are related; both have been linked with synaptogenesis. However, the time of peak brain growth does not correspond exactly with the occurrence of NCD and synaptogenesis for DA neurons in rats or monkeys.

The present data show that in fetal monkey brains, a distinct peak of apoptotic NCD occurs in midbrain DA neurons at about E80, midway through gestation. We estimate that at least 50% of the population is lost in this 25-day period. Other data reported here suggests that the onset of apoptosis in DA neurons is linked with the time of greatest rate of increase in striatal DA innervation. This is consistent with the theory that apoptosis in this population is triggered by inadequate supply of striatal growth factors.

Acknowledgments

We thank the staff of St. Kitts Biomedical Foundation, Feng-Pei Chen and Dottie Cameron for their excellent work. Supported by NS40570 and NS044281.

References

- Bayer SA, Altman J, Russo RJ, Zhang X. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. *Neurotoxicology*. 1993; 14:83–144. [PubMed: 8361683]
- Bessert DA, Skoff RP. High-resolution in situ hybridization and TUNEL staining with free-floating brain sections. *J Histochem Cytochem*. 1999; 47:693–702. [PubMed: 10219061]
- Brand S, Rakic P. Cytodifferentiation and synaptogenesis in the neostriatum of fetal and neonatal rhesus monkeys. *Anat Embryol*. 1984; 169:21–34. [PubMed: 6721218]
- Bundock EA. Doctoral Thesis. Department of Neuroscience, The Chicago Medical School; Chicago: 1999. Development of mesencephalic dopamine neurons in the African green monkey; p. 134
- Bursch W, Kleine L, Tenniswood M. The biochemistry of cell death by apoptosis. *Biochem Cell Biol*. 1990; 68:1071–1074. [PubMed: 2257115]
- Carvey PM, Punati A, Newman MB. Progressive dopamine neuron loss in Parkinson's disease: the multiple hit hypothesis. *Cell Transplant*. 2006; 15:239–250. [PubMed: 16719059]
- Chan WY, Yew DT. Apoptosis and Bcl-2 oncoprotein expression in the human fetal central nervous system. *Anat Rec*. 1998; 252:165–175. [PubMed: 9776071]
- Clancy B, Darlington RB, Finlay BL. Translating developmental time across mammalian species. *Neuroscience*. 2001; 105:7–17. [PubMed: 11483296]
- Clarke, P. Apoptosis versus necrosis. In: Koliatsos, V.; Ratan, R., editors. *Cell Death and Diseases of the Nervous System*. Humana Press; Totowa, New Jersey: 1999. p. 3-28.
- Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Human Dev*. 1979; 3:79–83.
- Elsworth JD, Deutch AY, Redmond DE Jr, Taylor JR, Sladek JR Jr, Roth RH. Symptomatic and asymptomatic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated primates: biochemical changes in striatal regions. *Neuroscience*. 1989; 33:323–331. [PubMed: 2622529]
- Elsworth JD, Morrow BA, Roth RH, Redmond DE Jr. Differential sensitivity to MPTP during the prenatal and neonatal development periods in primates. *Soc Neurosci*. 2005 Abstr. 897.817.
- Fearnley JM, Lees AJ. Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain*. 1991; 114(Pt 5):2283–2301. [PubMed: 1933245]
- Forno LS. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol*. 1996; 55:259–272. [PubMed: 8786384]
- Goldman-Rakic PS. Prenatal formation of cortical input and development of cytoarchitectonic compartments in the neostriatum of the rhesus monkey. *J Neurosci*. 1981; 1:721–735. [PubMed: 7346581]
- Hattori T, McGeer PL. Synaptogenesis in the corpus striatum of infant rat. *Exp Neurol*. 1973; 38:70–79. [PubMed: 4120090]
- Howard, CV.; Reed, MG. *Unbiased Stereology: Three Dimensional Measurement in Microscopy*. Springer-Verlag; New York: 1998.
- Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem*. 1981; 29:577–580. [PubMed: 6166661]
- Hu Z, Yuri K, Ozawa H, Lu H, Kawata M. The in vivo time course for elimination of adrenalectomy-induced apoptotic profiles from the granule cell layer of the rat hippocampus. *J Neurosci*. 1997; 17:3981–3989. [PubMed: 9151715]
- Ikonomidou C, Bittigau P, Koch C, Genz K, Hoerster F, Felderhoff-Mueser U, Tenkova T, Dikranian K, Olney JW. Neurotransmitters and apoptosis in the developing brain. *Biochem Pharmacol*. 2001; 62:401–405. [PubMed: 11448448]

- Jeon BS, Kholodilov NG, Oo TF, Kim SY, Tomaselli KJ, Srinivasan A, Stefanis L, Burke RE. Activation of caspase-3 in developmental models of programmed cell death in neurons of the substantia nigra. *J Neurochem.* 1999; 73:322–333. [PubMed: 10386985]
- Kholodilov N, Yarygina O, Oo TF, Zhang H, Sulzer D, Dauer W, Burke RE. Regulation of the development of mesencephalic dopaminergic systems by the selective expression of glial cell line-derived neurotrophic factor in their targets. *J Neurosci.* 2004; 24:3136–3146. [PubMed: 15044553]
- Kuan CY, Roth KA, Flavell RA, Rakic P. Mechanisms of programmed cell death in the developing brain. *Trends Neurosci.* 2000; 23:291–297. [PubMed: 10856938]
- Marti MJ, James CJ, Oo TF, Kelly WJ, Burke RE. Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra. *J Neurosci.* 1997; 17:2030–2039. [PubMed: 9045731]
- Moller A, Strange P, Gundersen HJ. Efficient estimation of cell volume and number using the nucleator and the disector. *J Microscopy.* 1990; 159:61–71.
- Morrow BA, Elsworth JD, Roth RH. Fear-like biochemical and behavioral responses in rats to the predator odor, TMT, are dependent on the exposure environment. *Synapse.* 2002; 46:11–18. [PubMed: 12211094]
- Morrow BA, Redmond DE Jr, Roth RH, Elsworth JD. Development of A9/A10 dopamine neurons during the second and third trimesters in the African green monkey. *J Comp Neurol.* 2005; 488:215–223. [PubMed: 15924344]
- Onn SP, Berger TW, Stricker EM, Zigmond MJ. Effects of intraventricular 6-hydroxydopamine on the dopaminergic innervation of striatum: histochemical and neurochemical analysis. *Brain Res.* 1986; 376:8–19. [PubMed: 3087584]
- Oo TF, Burke RE. The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra. *Brain Res Dev Brain Res.* 1997; 98:191–196.
- Oo TF, Kholodilov N, Burke RE. Regulation of natural cell death in dopaminergic neurons of the substantia nigra by striatal glial cell line-derived neurotrophic factor in vivo. *J Neurosci.* 2003; 23:5141–5148. [PubMed: 12832538]
- Oppenheim RW. Cell death during development of the nervous system. *Annu Rev Neurosci.* 1991; 14:453–501. [PubMed: 2031577]
- Ramsden DB, Parsons RB, Ho SL, Waring RH. The aetiology of idiopathic Parkinson's disease. *Mol Pathol.* 2001; 54:369–380. [PubMed: 11724911]
- Rehn AE, Rees SM. Investigating the neurodevelopmental hypothesis of schizophrenia. *Clin Exp Pharmacol Physiol.* 2005; 32:687–696. [PubMed: 16173923]
- Sladek, JR., Jr; Blanchard, B.; Collier, TJ.; Elsworth, JD.; Taylor, JR.; Roth, RH.; Redmond, DE, Jr. Development of mesencephalic dopamine neurons in the nonhuman primate. In: Bloom, FE.; Kupfer, DJ., editors. *Psychopharmacology: The Fourth Generation of Progress.* Raven; New York: 1995. p. 269-282.
- Weinberger DR. Implications of normal brain development for the pathogenesis of schizophrenia. *Arch Gen Psychiatry.* 1987; 44:660–669. [PubMed: 3606332]
- Wilson JM, Levey AI, Rajput A, Ang L, Guttman M, Shannak K, Niznik HB, Hornykiewicz O, Pifl C, Kish SJ. Differential changes in neurochemical markers of striatal dopamine nerve terminals in idiopathic Parkinson's disease. *Neurology.* 1996; 47:718–726. [PubMed: 8797470]
- Wong DF, Harris JC, Naidu S, Yokoi F, Marengo S, Dannals RF, Ravert HT, Yaster M, Evans A, Rousset O, Bryan RN, Gjedde A, Kuhar MJ, Breese GR. Dopamine transporters are markedly reduced in Lesch-Nyhan disease in vivo. *Proc Natl Acad Sci U S A.* 1996; 93:5539–5543. [PubMed: 8643611]

Figure 1.

Histological evidence of apoptosis in developing primate midbrain during the period of natural cell death. A, chromatin clumping (blue-purple) in a remarkably intact, orange-brown, tyrosine hydroxylase-immunoreactive cell (see +), counterstained with thionin (blue-purple); B, more usually observed chromatin clumping (blue-purple) in orange-brown, tyrosine hydroxylase-immunoreactive cell (see +); C, anti-ACTIVE caspase-3 immunoreactivity (black); D, discrete TUNEL labeled body (orange-brown) containing thionin stained chromatin clumping (see #); E, a more diffuse TUNEL staining (orange-brown, see *) in ventral tegmental area with TH-ir cells (black); and F, diffuse TUNEL staining (orange-brown, see *) with TH-ir cells (black) in the substantia nigra. Bar is 10 microns for panels A, B, C, and D. Bar is 25 microns for panels E and F.

Figure 2.

The frequency of apoptotic midbrain dopamine neurons peaks at about E80 during fetal development in the monkey. Apoptosis was defined as the appearance of chromatin clumping in TH+ cells of the A9 and A10 regions.

Figure 3.

The progressive rise in striatal dopamine concentration through the prenatal, neonatal and adult stages of life in the African green (vervet) monkey. For the prenatal stage of development (left) each point represents one fetus, for a total of 11. Mean values $\pm$ standard error are shown for the postnatal data (right). For the neonatal period there were 5 samples (range 11-25 postnatal days), and the adult values derive from 11 untreated animals.