Animal Models of Induced Apoptotic Death in the Substantia Nigra

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1. Introduction

Apoptosis is a form of cell death in which genetically regulated programs intrinsic to the cell bring about its own demise. In recent years, there has been a tremendous growth of interest in apoptosis as a mechanism of disease in a wide range of human disorders including the neurodegenerative diseases, such as Parkinson's disease (PD) (1). This growth of interest has spawned an extraordinary number of recent discoveries about the molecular basis of apoptosis. It is important to emphasize, however, that much of this new knowledge has been attained in the study of relatively simple systems, such as invertebrate models or mammalian nonneural cell culture systems. Less is known about these mechanisms in neural cells, and much of what is known is based on studies of peripheral neural cells (such as sympathetic ganglia and PC12 cells) in tissue culture. Much less is known about central neurons; in particular, we know little about the regulation of apoptotic death in central neurons in living animals. It is especially important to try to identify the mechanisms of cell death in central neurons of known phenotype, particularly those implicated in human neurodegenerative disease, such as the dopamine neurons in PD. The purpose of the models we have developed of induced apoptosis in dopamine neurons of the substantia nigra (SN) is to try to translate what is being learned about the molecular mechanisms of apoptosis in simpler systems to these neurons.

Thus far, our studies of these models have shown, as would be expected, that there are important major parallels between the regulators of cell death in dopamine neurons and those identified in many simple mammalian cells. Nevertheless, a continued detailed analysis of these regulators in dopamine neu-
rons is important, because there are now many precedents for differences in the mechanisms of apoptosis between different cell phenotypes, and even within a phenotype, depending on the nature of the death stimulus. Greene and his colleagues have shown that within postmitotic sympathetic ganglion neurons, the particular mechanisms of apoptosis differ when death is induced by trophic factor withdrawal, oxidative stress, and DNA damage (2).

The models we describe in this chapter are developmental models. We have shown that during normal development, a natural cell death event with the morphology of apoptosis occurs within the dopamine neurons of the SN (3,4). It would be predicted by classic neurotrophic theory that the magnitude of this death event would be regulated by early target interactions (5,6), and we have shown that this is the case; destruction either of the striatal target with the excitotoxin quinolinic acid (QA) (7) or of intrastriatal dopamine terminals with the neurotoxin 6-hydroxydopamine (6-OHDA) (8) results in an approximately tenfold increase in the number of apoptotic profiles in the SN pars compacta (SNpc) (Fig. 1). In both of these models, apoptotic death has been identified within phenotypically defined dopamine neurons, and the apoptotic nature of the death process has been confirmed by ultrastructural and end-labeling (TUNEL) criteria.

The question arises as to whether developmental models of apoptosis are likely to be relevant to the cell death processes of adult-onset, neurodegenerative disorders, such as PD. At the core of the hypothesis that apoptosis may underlie adult-onset neurodegenerative disease is the concept that the genetic programs mediating apoptosis, which are normally active during development, become abnormally reactivated in the adult. Thus the study of mechanisms of apoptosis in a particular neuronal phenotype during development is entirely germane to the hypothesis that apoptosis may play a role in adult neurodegenerative disorders. Under no circumstance of neuron death in the adult brain (where apoptosis has been postulated to play a role) has it been proposed that an adult mechanism of apoptosis may be separate and distinct from those occurring in the developmental setting. On the contrary, it has been assumed that these processes are highly related, if not identical, and this assumption has led to numerous important discoveries of the mechanisms of apoptosis across species and developmental ages.

Horvitz and his colleagues made the assumption that the regulation of developmental cell death in the nematode *Caenorhabditis elegans* is related to the regulation of apoptosis in other biologic contexts; this led to important insights into the functional role of Bcl-2 as a negative regulator of apoptosis, and to the discovery of the caspases (9). Indeed, most of what we now know about the molecular basis of apoptosis derives from studies of developmental models. Such models include nerve growth factor (NGF) withdrawal from neonatal
sympathetic ganglia in culture, characterized by Johnson and coworkers (10) and others; withdrawal of serum from premitotic PC12 cells, or NGF withdrawal from postmitotic PC12 cells, characterized by Greene and colleagues (11) and others (12); withdrawal of serum and potassium from postnatal cerebellar neurons (13); naturally occurring developmental cell death in motorneurons in chick, studied extensively by Oppenheim (14) and others; developmental degeneration of intersegmental muscles in hawkmoth, characterized by Schwartz and colleagues (15); and normal cell death in Drosophila, studied by Steller and others (16). We therefore propose that the study of the mechanisms of apoptosis in living dopamine neurons during development will provide useful knowledge about its regulation in other contexts, including that of disease in the adult brain.

We have used these models primarily to study the molecular correlates of apoptosis within dopamine neurons at a cellular level, using anatomic techniques (17–19). They also can be used for a regional analysis of the biochemical correlates of cell death. The striatal lesion model, induced by QA injection (Fig. 2), induces an increase in apoptotic death in SN that peaks by 24 h post
lesion and abates by 96 h (7). The intrastriatal 6-OHDA injection model results in a complete loss of dopaminergic terminals in the striatum (Fig. 3A), with complete preservation of intrinsic striatal neurons (Fig. 3B). In this model, induced death in the SN is delayed in its onset, beginning on the third postlesion day, and is more protracted in its course, continuing until postlesion d 10 (8). This particular model is more selective for dopamine neurons, which, for some research questions, is an advantage. On the other hand, it is more destructive than the QA model; very few dopamine neurons survive. For this reason, the QA model may be more useful if one wishes to examine compensatory responses in surviving dopamine neurons.

2. Materials

2.1. Striatal Lesion with Quinolinic Acid

1. QA (Sigma, St. Louis, MO) is stored under desiccation at −20°C.
2. The vehicle for the QA injection is phosphate-buffered saline (PBS) 0.1 M (pH 7.1)/0.9% NaCl.

Fig. 2. Nissl stain of a coronal section of the striatum on postlesion d 2 following intrastriatal injection of QA on PND 14. Neuron loss is indicated by regions of pallor. It can be seen that there is pallor throughout the striatum on the experimental (E) side. Note that the lesion, while predominantly within the striatum, is not restricted to it, as there is also some pallor in the overlying cortex and in the adjacent medial septal area. C, control side.
Fig. 3. Coronal sections of the striatum following intrastriatal injection of 6-OHDA. (A) Tyrosine hydroxylase (TH) immunostaining to demonstrate dopaminergic fibers within the striatum. Normal TH-positive fiber staining appears black, with an increasing medial-to-lateral gradient on the control (C) side. On the experimental (E) side, the striatum is devoid of staining. (B) Nissl stain demonstrating that there is no loss of intrinsic striatal neurons on the E side following the 6-OHDA lesion.

3. Anesthesia is induced by hypothermia alone (see Note 1a).
4. The intrastriatal QA infusion is performed with a CMA 100 microinfusion pump (Carnegie Medicin, Stockholm, Sweden).
5. Blue tubing connectors, fluorinated ethylene propylene (FEP) tubing, a cannula clip, and 500-μL syringes (Bioanalytical Systems, West Lafayette, IN).
6. A 28-gage cannula (Plastic One Products, Roanoke, VA). We cut the cannulae down to a 4-mm length in our lab. The cannula is held by the clip mounted on the arm of a David-Kopf stereotaxic apparatus and connected to the syringe using FEP tubing with blue tubing connectors.
7. A Dremel hand-held drill is used to make a skull burr hole.
8. After surgery, animals recover in a Thermo Care small animal incubator (Harvard Apparatus).

2.2 Dopaminergic Terminal Lesion with 6-OHDA
1. 6-OHDA-HBr (Regis, Morton Grove, IL), stored under nitrogen in a desiccator at −20°C.
2. L- ascorbic acid (Sigma), stored at room temperature.
3. Desmethylimipramine hydrochloride (DMI; Sigma), stored at 4°C.

2.3. Substantia Nigra Microdissection for Molecular and Biochemical Studies
1. A mouse brain matrix (30 g, coronal; Harvard Apparatus).
2. Large and small scissors and blunt and hooked forceps (Harvard Apparatus).
3. Petri dishes, spatula, razor blades, and sterile cryogenic vials (Nalgene; Fisher Scientific).

3. Methods
3.1. QA Injection
1. Timed pregnant rats (Sprague-Dawley; Charles River, Wilmington, MA) (see Note 1). We order multiple pregnancy females, i.e., multiparous females. These animals are more likely than nulliparous females to have larger litters and to nurse successfully.
2. Inspect the cage in the afternoon of each day; day of birth is defined as postnatal day (PND) 1.
3. Bring the pups into the lab on the day of the experiment and keep them at 33°C in the small animal incubator, separated from the dam. PND 7 pups are used for molecular and biochemical studies; PND 14 pups are used for anatomic studies for greater ease of tissue processing (see Note 2).
4. To prepare QA for injection, add 80.2 mg for each 1 mL of cold 0.1 M PBS and keep on ice. Add 70 μL of 10 N NaOH and mix thoroughly to dissolve the QA. Check pH with pH paper (range 6.0–8.0). Then, add 10 μL of 1 N NaOH stepwise to achieve pH 6.8. This solution, 480 mM, is stable for 3 d at 4°C.
5. Weigh each pup and anesthetize with hypothermia.
6. Load the 500-μL syringe with the QA solution and flush the tubing and cannula before the injection.
7. Place the anesthetized pup in the prone position on a ceramic plate, which has been stored at −20°C. Hypothermia provides additional anesthesia.
8. On the plate, support the chin with four glass microscope slides. It is essential to keep the chin level on glass slides to provide a correct and reproducible dorsal-ventral angle between the neck and the snout.
9. Place the pup and chilled plate on a Plexiglas stage.
10. Under a dissecting microscope, expose the skull by a midline incision, and drill a burr hole at 3.0 mm lateral to the left of bregma on the coronal suture.
11. Then move the Plexiglas stage onto the base platform of the stereotaxic apparatus toward the cannula holder.
12. Position the 28-gage cannula directly above the burr hole.
13. Pierce the dura with a needle and then insert the cannula, 4.0 mm vertically, into the striatum using the Kopf vertical adjustment knob.
14. Infuse QA at a rate of 0.50 μL/min for 2 min.
15. Slowly withdraw the cannula 2 min after the end of the infusion.
16. Suture the scalp with silk 5-0.
17. Inject PBS as a vehicle control.
18. We allow pups to recover in the small animal incubator at 33°C for 4 h, but the precise temperature should be determined empirically for each lab (see Note 3).
19. While animals are in the incubator, record their progress and behavior. As animals recover from anesthesia, they initially will rotate away from the side of the QA lesion. Eventually, rotational behavior stops, and animals will show normal motor and nursing behavior.
20. At the end of 4 h, return pups to the dams until the assigned postlesion day.

3.2. 6-OHDA Unilateral Injection

1. For the DMI injection, prepare 2.5 mg of DMI in 1 mL cold 0.9% NaCl.
2. Weigh the pups and treat them with 25 mg/kg DMI, subcutaneous interscapular injection, 30 min before 6-OHDA lesion (see Note 4).
3. In a test tube wrapped in aluminum foil, dissolve 15 mg 6-OHDA in 1 mL cold 0.9% (w/v) NaCl containing 0.02% (w/v) ascorbic acid (see Note 5). The solution must be clear. Any sign of brownish discoloration indicates that the 6-OHDA has oxidized, and it cannot be used.
4. Draw 500 μL of the solution into a syringe wrapped in foil and store the remaining volume on ice (see Note 6).
5. Flush the tubing and the cannula with fresh 6-OHDA before the injection.
6. Anesthetize the pup by inducing hypothermia.
7. Place the pup in the prone position on a chilled plate on a Plexiglas stage, with its chin resting on four glass slides as described above.
8. Under a dissecting microscope, expose the skull by a midline incision; drill a burr hole at 3.0 mm lateral to the left of bregma on the coronal suture.
9. The Plexiglas stage is moved onto the base platform of the stereotaxic apparatus toward the cannula holder, where a 28-gage cannula is inserted 4.0 mm vertically into the striatum as described above.
10. Infuse the 6-OHDA at a rate of 0.25 μL/min for 4 min.
11. Slowly withdraw the cannula 2 min after the end of the infusion.
12. Suture the scalp with Silk 5-0.
13. Flush the cannula and tubing before continuing onto another pup. Inject saline/0.02% ascorbate as vehicle control.
14. Allow the pups to recover in the small animal incubator for 4 h, and record their progress and behavior.
15. At the end of 4 h, return pups to the dams until the assigned postlesion day.

3.3. Substantia Nigra Microdissection for Molecular and Biochemical Studies

1. On the assigned postlesion day, using large scissors, remove the head of the rat pup.
2. Using scissors and forceps, remove the brain and place it into chilled 0.9% NaCl for approx 10 s.
3. Using the forceps, place the brain ventral surface down on a Petri dish on ice and remove the region caudal to the colliculi.
4. Again, with hooked forceps, place the brain into the mouse brain matrix with the ventral surface upward.
5. Mark both sides of each razor, to indicate the experimental side and avoid any possibility of left/right confusion.
6. With the ventral surface up, the left (experimental) side of the brain is on your right; thus mark the right side of each razor blade with an “E.” Rest one razor blade in the third posterior slot. Rest a second razor in posterior slot 5. The blades should be just anterior (#5) and just posterior (#3) to the midbrain.
7. Push the blades into the brain at the same time.
8. Using a spatula, remove areas of the brain anterior and posterior to the razor blades.
9. Remove the blade in slot 5.
10. Using the spatula, pull the dorsal region of the brain from the edge of the block onto the second blade.
11. Remove the razor supporting the brain section from slot 3, and place the section on a Petri dish, keeping the same orientation.
12. Using forceps, dissect away and discard the cortices and mamillary body.
13. Using the razor blade, make a horizontal cut just below the cerebral aqueduct and discard the dorsal half of the section.
14. Then, with a vertical cut, split the remaining section in half. The portion on your left will be the right (control) side of the brain (see Fig. 4). The portion on your right will be the left (experimental) side.
15. Place each SN piece into appropriately labeled vials on dry ice. Each SN will weigh about 20 mg. The SNs can be stored at –80°C for 6 mo.

4. Notes

1. Although timed pregnant animals are somewhat more expensive than nontimed ones, we have found that the predictability of timed pregnancies, and the improved efficiency of planning experiments, make them well worth the additional expense.
1a. In our publications on these models, we specify that we use Metofane (Schering-Pough) for induction of anesthesia. However, Metofane is no longer available in the United States. For this reason we now anesthetize with hypothermia alone. Hypothermia provides a very effective, lasting anesthesia and is compatible with induction of both the QA and the 6OHDA models.
Fig. 4. Schematic of the mesencephalon demonstrating landmarks for substantia nigra (SN) microdissection. The schematic depicts a 2.0-mm-thick section lying anterior surface up. The overlying cortex is not shown. We remove the mamillary body with forceps. We then cut the section horizontally (dotted line) just below the aqueduct and discard the dorsal piece. The ventral piece is then cut vertically (dotted line) in the midsagittal line to separate the left and right SN. Note that the "SN" piece contains not only SN, but also an overlying region of the central mesencephalon.

2. We have previously shown that the magnitude, distribution and morphology of cell death in SN is identical between PND 7 and PND 14 (20).

3. In our experience, a major potential source of variability in the striatal QA lesion model is the postoperative temperature of the animals. It has been demonstrated that body temperature does influence the magnitude of lesions made by excitotoxins (21). For this reason, we would suggest that any investigator who uses the striatal QA lesion model should, in their own lab, investigate the influence of different postoperative temperatures on the extent of the striatal lesion.
Although the striatum lies at a considerable distance from the SN, it is possible, if postoperative temperature is too high, for the QA injection actually to cause direct damage to the SN, leading to misleading results.

4. 6-OHDA, a light-sensitive compound, oxidizes easily at room temperature. Prepare 6-OHDA 15 min before the injection.

5. Note that NaCl must be used to dissolve 6-OHDA; in PBS it oxidizes immediately.

6. In our experience, 6-OHDA solution is stable for 1 h at room temperature. After this time, replenish the working solution to avoid injecting oxidized solution.

Reference


