Experimental Neurobiology
Vol. 12, pages 127~133, December 2003

Changes of Calbindin-D_{28k}-containig Neurons on the Substantia Nigra of 6-Hydroxy Dopamine Induced Parkinsonian Rat Models

Young-Bok Yoo*

Department of Anatomy, College of Medicine,
Dankook University, Cheonan, Korea

ABSTRACT

Parkinson's disease is the motor disorder which results from the selective degeneration of the dopaminergic neurons in the substantia nigra (SN) and the deficiency of dopamine in the corpus striatum (CS). But etiological factors are still unknown. In this study, we made parkinsonian rat models which were under oxidative stress injecting 6-OHDA in SN. According to the time transition after neurotoxin injection we made the coronal sections of SN immunohistochemical staining by anti-Tyrosine hydroxylase (TH) and calbindin-D_{28K} antibody. We compared the survival rate (%) of calbindin-D_{28K}-immunoreactive cells with that of TH-immunoreactive cells to confirm the suggested role of calbindin-D_{28K} which may protect dopaminrgic neurons against the cytotoxicity according to the time transition after 6-OHDA injection. In parkinsonian rat models which were sacrificed 7 d, 10 d, 14 d, 17 d after the injection of 6-OHDA, the survival rates of calbindin-D_{28K}-immunoreactive neurons is significantly higher than that of TH-immunoreactive neurons ($p > 0.05$). We suggest that calbindin-D_{28K} containing neurons tend to be spared during the acute period of parkinsonian rat models, calbindin-D_{28K} helps to protect neurons against excitotoxicity. This is consistent with the proposed calbindin-D_{28K} function as an intraneuronal calcium buffer.

Key words: Calbindin-D_{28K}, TH, Parkinson's disease, substantia nigra, rat

INTRODUCTION

Parkinson's disease (PD) is an idiopathic neurodegenerative disorder that progresses clinically over years to decades. The main feature of PD responsible for its motor manifestation is progressive degeneration of dopaminergic neurons in the substantia nigra, and a concomitant loss of DA in the striatum. The cellular mechanisms underlying the protracted clinical progression of PD are not known (Fearnley and Lees, 1991; Jenner et al., 1992; Jenner and Olanow, 1996).

Pathologic studies have reported the presence of many atrophic neurons in the substantia nigra, implying that the time course of neuronal degeneration may be a protracted and gradual, rather than an acute process. These observations provide a basis for putative neuroprotective therapies that attempt to prolong the survival and stimulate the recovery of slowly degenerating neurons (Lindsay et

*To whom correspondence should be addressed.
TEL: 041-550-3856, FAX: 041-550-3905
e-mail: ybyoo36@hanmail.net
The present research was conducted by the research fund of Dankook University in 2003.

al., 1993; Parkinson study group 1993; Koller 1997).

Calbindin-D_{28K} is a member of the EF-hand family of calcium binding proteins and is extensively used as neuronal markers, since these soluble proteins fill the cytoplasm of neuronal processes and so facilitate studies of neuronal shape, connectivity and functional specialization (Baimbridge et al., 1992). Many reports were currently investigating the role of calbindin-D_{28K}, in the brain relative to neurodegerative disease. Calbindin-D_{28K} is found in many major cell groups and fiber tracts throughout the brain (Jande et al., 1981; Baimbridge et al., 1982; Rogers, 1992). Calbindin-D_{28K} is believed to function as an intraneuronal calcium buffer preventing accumulation of excessive levels of cytosolic free Ca^{2+}.

Recent human and animal data have indicated that there is a reduced gene expression of calbindin-D_{28K} in those brain areas affected by normal aging and in Parkinson's, Huntington's, and Alzheimer's diseases (Iacopino et al., 1992).

In Parkinson's disease a dramatic reduction in calbindin-D_{28K} mRNA and protein levels is found in the substantia nigra (Anthony et al., 1990), but the pigmented neurons that are immunoreactive to calbindin-D_{28K} in the substantia nigra of these patients seem to be selectively spared from degeneration (Yamada et al., 1990). In PD monkeys, midbrain DA neurons containing calbindin-D_{28K} are relatively spared compared to those devoid of this protein (Lavoy and Parent, 1991).

Calbindin-D_{28K} gene expression may be a critical factor which determines neuronal survival in the substantia nigra during Parkinson's disease. It is possible that reduced calbindin-D_{28K} gene expression in these key brain areas leads to excitotoxic vulnerability and Ca^{+2}-mediated neuronal degeneration (Yamada et al., 1990; German et al., 1992)

To date, the charaterization of animal models has been based more on biochemical measures or responses to behavioral tests rather than precise cellular and morphological identification and analysis. There has been a paucity of information concerning the specific neurons or neuronal cell groups affected by the models as well as their similarity to the human condition. Relatively little is known about the discrete neuronal populations which are affected by excitotoxins.

PD does not occur spontaneously in animals, which means that experimental nigrostriatal degeneration can be induced only by neurotoxic or surgical lesions. The most widespread animal models of PD use intracerebral injections of the catecholamine cell-specific neurotoxin 6-hydroxydopamine (6-OHDA)(Perese et al., 1989; Sauer and Oertel, 1994; Lee et al., 1996) systemic administration of N-methyl-4-pheny-1,2,3,6-tetrahydropyridine (NPTP) (Lavoy and Parent, 1991; Germann et al., 1992) Rats are generally resistant to MPTP, 6-OHDA is commonly used to create hemiparkinsonian rats. When this toxin is injected into the dopaminergic fiber bundle at the rostromedial aspect of the substantia nigra unilaterally (Perese et al., 1989)

The objectives of this study were to compare the neuronal calbindin-D_{28K} status in 6-OHDA induced parkinsonian rat models with what was previously observed in the human condition and animal models to provide information concerning calbindin-D_{28K}-containing neuronal populations according to the time transition after 6-OHDA injection.

MATERIALS AND METHODS

Animals

The animals studied were male normal Sprague-Dawley rats (Taconic Farms) weighing 270～300 g at the start of the experiment. They were housed 6 to a cage with free access to rat chow and water and kept in a cyclically illuminated environment.

6-OHDA induced parkinsonian rat models

The animals were anesthetized with chloral hydrate, 350 mg/kg i.p., and secured in a Naarishige stereotaxic frame (Narishige Scientific Instrument laboratory, Tokyo, Japan) with the bite bar 3.3 mm below the horizontal. A 2-㎍/㎕ solution of 6-OHDA (Sigma Chemical Co.) was prepared with ascorbic acid (0.2 mg/ml)(Sigma Chemical Co.) added to prevent auto-oxidation. This solution was kept on ice until it was injected.

A dental drill was used to place a single burr hole at the appropriate site on the right. Selective lesions were created by injecting 2㎕ of 6-OHDA at 1㎕/min using a Hamilton 10㎕ syringe with a 26-gauge needle. Two separate sites were injected.

The initial injection was made with the needle bevel directed rostrally at the following coordinates:

anterior 3.5 mm, lateral 1.9 mm, dorsoventral 7.1 mm with respect to lambda and dura, based on the Paxinos and Watson Stereotaxic Atlas (1986). The second injection was made with the needle bevel directed laterally at the following coordinated: anterior 3.5 mm, lateral 2.3 mm, and dorsoventral 6.8 mm. At the completion of each injection, the needle was left in place for 3 min and then withdrawn at 1 mm/min. The Burr hole was filled with gelatin sponge (Gelfoam-Upjohn Co., Kalamazoo, MI) and the skin closed (Perese et al., 1989).

One control group received 2-μl injections of normal saline instead of 6-OHDA in the manner described above.

Rotational testing

The animals were tested for rotational behavior induced by 5 mg/kg amphetamine (i.p.), starting 6 d after 6-OHDA injection. Their turning was measured for 90-min periods approximately every two weeks with a minimum of 48 h between drugs for a total of 12 mo. The number of full clockwise turns/min in response to amphetamine was recorded. Animals demonstrating an average of at least 7 clockwise turns/min over 90 min in response to amphetamine were then selected for further study.

Histology

Representative parkinsonian rat models fulfilling the rotational driteria described above were sacrificed 7 d, 10 d, 14 d, 17 d, 4 weeks, 6 weeks, 3 mo, 5 mo, 7 mo, 12 mo after 6-OHDA injection. The animals were sacrificed with an overdose of chloral hydrate and perfused through the ascending aorta with 200 cc PBS followed by 200 cc of 10% formalin in PBS (pH 7.0). Their brains were rapidly removed and were then postfixed for 24 h in the same fixative. 30μm coronal sections were then cut on cryostat (Reichert Frigocut, model 2000), mounted on chrome-alum slides, and processed for immunohistochemistry. Brain sections were exposed for 24 h at 4°C to rabbit serum containing antibody to TH (Eugene Tech Inernational, 1 : 1000) and calbindin-D_{28K} (Sigma Chemical Co., 1 : 300) containing 0.3% Triton X-100 and 1% normal goat serum respectively. The sections were washed 3 times (10 min) and then incubated for 60 min in biotinylated goat secondary. The sections were washed again and incubated for 60 min in vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). They were washed in PBS (pH 7), developed in diaminobenzidine tetrahydrochloride (DAB) for 1～5 min, washed in tap water, cleared, and covered.

Light microscopic observation

Representative parkinsonian rat models exhibiting the stable turning behavior described above were sacrificed 7 d, 10 d, 14 d, 17 d, 4 weeks, 6 weeks, 3 mo, 5 mo, 7 mo, 12 mo after 6-OHDA injection.

We counted the TH- and calbindin-D_{28K}-immunoreactive cells in the normal and lesioned side of the SN respectively under light microscope (X200) attached with square eyepiece micrometer (Olympus). The mean±S.E. was calculated from the cell counts of the three coronal sections at bregma -5.2 mm based on Paxinos Atlas (1986).

The survival rate (%) was the value that the cell count of the lesioned side was divided by the cell count of normal side. According to the time transition after 6-OHDA injection we compared the survival rates of calbindin-D_{28K}-immunoreactive cells with those of TH-immunoreactive cells. We used Student's t-test to confirm the significant difference of the survival rates statistically.

RESULTS

Coronal sections through the all groups' midbrains after neurotoxin injection stained with cresy violet demonstrated a striking decrease of cell bodies in the lesioned substantia nigra. TH and calbindin-D_{28K} immunohistochemistry of additional sections through the same region revealed a striking decrease of the immunoreactive cells in the lesioned substantia nigra. Sections taken more rostrally demonstrated no TH reactivity of nerve fibers in the striatum ipsilateral to the lesioned nigra. Saline-lesioned animals demonstrated intact cell bodies in the substantia nigna with TH staining in the ipsilateral striatum equal to that of the normal side.

The survival rates of TH-immunoreactive cells was 46.0% at 7 d, 8.7% at 10 d, 8.3% at 14 d, 4.0% at 17 d, 5.7% at four weeks, 0% at six weeks. The survival rates of calbindin-D_{28K}-immunoreactive cells was 81.3% at 7 d, 55.3% at

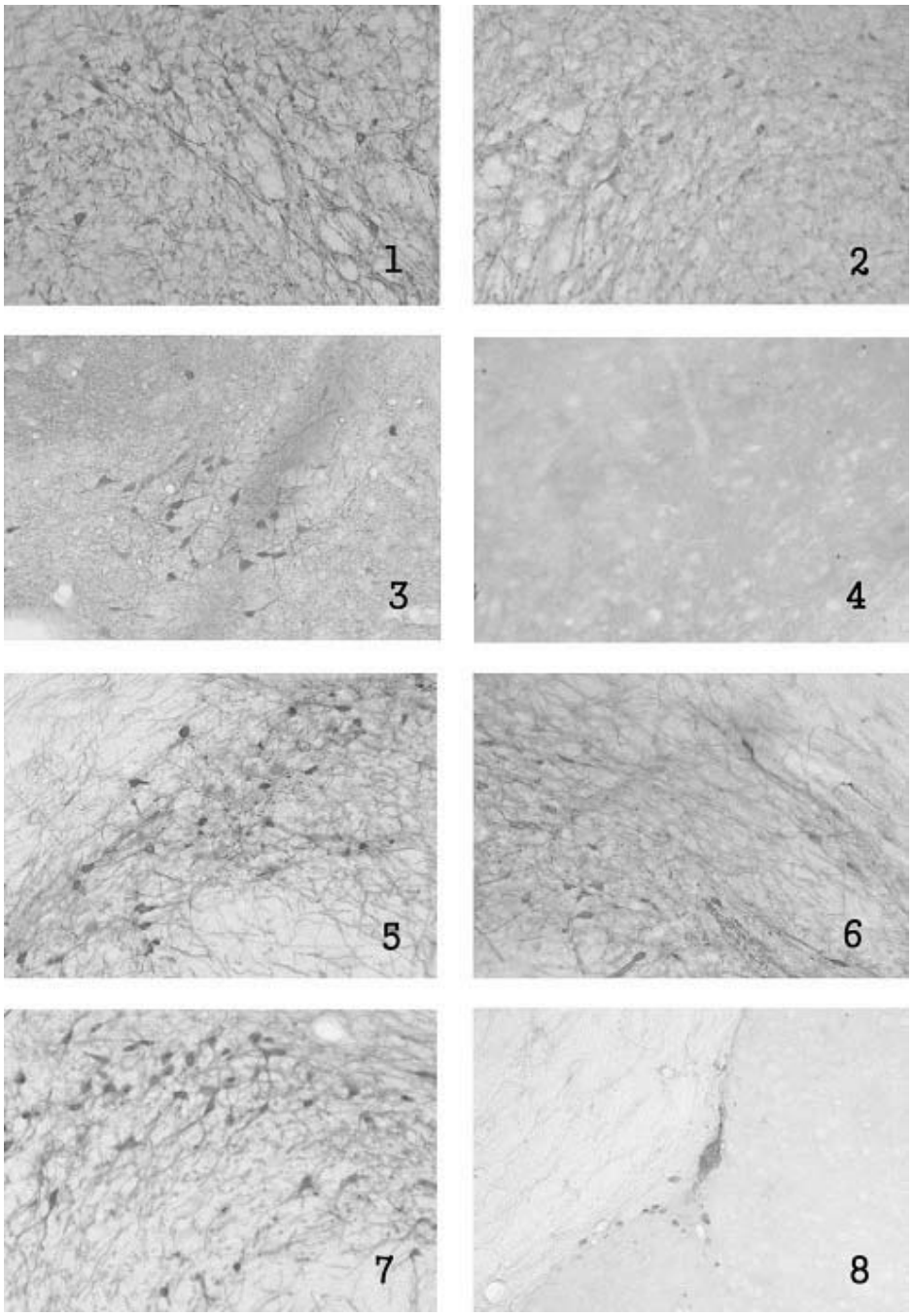

Fig. 1. 1. Calbindin-D_{28K} immunostaining of the lesioned SN in the parkinsonian rat model which was sacrificed one week after 6-OHDA injection. ×200. 2. Calbindin-D_{28K} immunostaining of the normal SN in the parkinsonian rat model which was sacrificed one week after 6-OHDA injection. ×200. 3. Calbindin-D_{28K} immunostaining of the lesioned SN in the parkinsonian rat model which was sacrificed seven months after 6-OHDA injection. ×200. 4. Calbindin-D_{28K} immunostaining of the normal SN in the parkinsonian rat model which was sacrificed seven months after 6-OHDA injection. ×200. 5. TH immunostaining of the lesioned SN in the parkinsonian rat model which was sacrificed one week after 6-OHDA injection. ×200. 6. TH immunostaining of the normal SN in the parkinsonian rat model which was sacrificed one week after 6-OHDA injection. ×200. 7. TH immunostaining of the lesioned SN in the parkinsonian rat model which was sacrificed seven months after 6-OHDA injection. ×200. 8. TH immunostaining of the normal SN in the parkinsonian rat model which was sacrificed seven months after 6-OHDA injection. ×200.

Table 1. Survival (%) and number of tyrosine hydroxylase-immunoreactive cells in normal & lesioned side in substantia nigra of 6-OHDA induced Parkinsonian rat models

	Calbindin-D_{28K}			Tyrosine hydroxylase		
	Normal	Lesion	Survival (%)	Normal	Lesion	Survival (%)
7 D	16.7±2.9	13.6±3.5	*81.3%±7.0	96.77±4.8	44.3±0.7	46.0%±1.5
10 D	13.7±2.6	7.3±0.9	*55.3%±5.3	78.0±14.0	7.0±1.7	8.7%±1.1
14 D	16.0±2.1	5.0±0.6	*32.7%±6.7	93.7±7.8	7.7±0.9	8.3%±1.0
17 D	18.5±3.5	3.0±1.0	*15.5%±2.5	86.5±10.5	3.5±0.5	4.0%±3.4
4 Wk	18.3±1.9	1.5±0.5	9.0%±3.5	90.0±4.6	4.8±2.2	5.7%±2.0
6 Wk	17.0±4.0	0	0%	93.0±2.0	0	0%
3 Mo	14.0±1.0	0.3±0.3	0%	86.0±3.5	0	0%
5 Mo	14.3±2.0	0.7±0.2	0%	85.0±5.3	2.7±2.7	0%
7 Mo	14.3±1.5	0.3±0.5	0%	84.3±8.3	1.0±1.0	1%
12 Mo	15.8±0.9	0.5±1.2	0%	86.3±7.3	0	0%

Values shown represent the mean±S.E. of cell counts made on three coronal sections taken at level corresponding to bregma -5.2 mm on Paxinos Atlas. Cell survivals for each neuronal population are indicated as percentage (%) of lesioned value/normal value. *Significant difference from TH survival ($p<0.05$)

10 d, 32.7% at 14 d, 15.5% at 17 d, 9.0% at four weeks, 0% at six weeks (Table 1). In the parkinsonian rat models which were sacrificed 7 d, 10 d, 14 d, 17 d after the injection of 6-OHDA, the survival rates of calbindin-D_{28K}-immunoreactive neurons is significantly higher than that of TH-immunoreactive neurons in the SN ($p>0.05$).

DISCUSSION

The Ca^{2+} message is converted into an intracellular response-in many cases by calcium binding proteins that are involved in a wide variety of activities, such as cytoskeletal organization, cell motility and differentiation, cell-cycle regulation, and Ca^{2+} buffering and transport. It might therefore be possible that altered levels of some cacium binding proteins could lead to an impaired calcium ion homeostasis in nerve cells and to pathological conditions (Heizmann and Braun, 1992).

Several research groups have now started to search for altered expression of calbindin-D_{28K} in affected brain regions of patients suffering from acute insults, such as stroke and epileptic seizures, and from chronic neurodegenerative disorders. The precise functional role of this protein is not yet known, though they are believed to have some calcium "buffering" properties (Baimbridge et al., 1992). As such, they may be important in buffering high levels of intracellular calcium, which would otherwise lead to excitotoxic cell damage. Many studies support this putative neuroprotective role for the calcium binding proteins (Yamada et al., 1990; German et al., 1992).

Our results showed that the survival rates of calbindin-D_{28K}-immunoreactive cells were statistically higher than those of TH-immunoreactive cells until 17 days after the injection of 6-OHDA. But all of the calbindin-D_{28K}-immunoreactive cells and TH-immunoreactive cells were destructed after 6 weeks. Seventeen days took the 90% of TH-immunoreactive cells to destruct, four weeks took the 90% of calbindin-D_{28K}-immunoreactive cells to destruct. 54% of TH containing neurons were dead and 20% of calbindin-D_{28K} containing neurons were dead at one week after 6-OHDA injection. The 80% of TH containing neurons and the 33% of calbindin-D_{28K} containing neurons which were surviving at 7 days were destructed three days later. The destruction of TH containing neurons progressed quickly but that of calbindin-D_{28K} containing neurons progressed slowly until 4 weeks.

We did not examine double immunostaining of calbindin-D_{28K} and TH. But considering that most of the calbindin-D_{28K} positive neurons were TH positive in the SN (Roger, 1992), we suggest that most of the calbindin-D_{28K}-immunoreactive cells which were surviving after 6-OHDA injection were dopaminergic. Our results is consistent to the report that dopaminergic neurons containing calbindin-D_{28K} are rel-

atively spared compared to those devoid of this protein in the PD and parkinsonian monkey (Yamada et al., 1990; Lavoy and Parent, 1991). From our results we suggest that a relationship may exist between calbindin-D_{28K}-containing neurons and Ca^{+2}-mediated excitotoxic events. It is possible that neurons which contain calbindin-D_{28K} are less susceptible to the excitotoxic degeneration in the acute stage of parkinsonian animal models. This is consistent with the proposed calbindin-D_{28K} function as an intraneuronal calcium buffer.

67% of the lesioned animals met our rotational criteria of >7 clockwise turns/min in response to amphetamine. The brains of animals failing to meet these criteria were examined histologically. In most cases, the needle tracts were visualized in the appropriate location. This was presumed to be secondary to inadequate delivery of the 6-OHDA.

The animal models failed to exhibit the changes in calbindin-D_{28K} which were seen in their human counterparts. This may be an indication that the various models do not completely mimick the human conditions. All the animal models utilized an acute insult by direct brain injection or systemic application of excitotoxic substances in order to mimic the behavioral and biochemical abnormalities observed in a particular neurodegenerative disease. Due to a sudden and massive toxic insult near the cell body, neuronal loss in the substantia nigra is instant and static rather than gradually progressive (Lee et al., 1996). It is indeed possible, that with acute insults neurons containing calbindin-D_{28K} tend to be spared. Thus calbindin-D_{28K} helps to protect neurons against excitotoxicity. However, neurodegenerative disorders in man do not occur in response to an acute insult and are chronic conditions.

Any analysis of animal models must make mention of the fact that there is likely to be a lack of the multiplicity of lesions and abnormalities present in the human condition. These multiple problems are probably very important in the overall development of the pathological processes. There is always the possibility of confounding results generated by damage to nearby fibers or cells which goes undetected (Iacopino et al., 1992) Given the apparent complexity of the neurodegenerative diseases and their clinical manifestations, it seems reasonable to consider that simultaneous degeneration of a number of brain regions or the existence of a number of different pathologies may be required for their development and ultimate progressions. It is unreasonable to expect animal studies to provide definitive evidence for the mechanisms underlying these investigations, when combined with human data, should ultimately contribute to a more complete understanding.

REFERENCES

Anthony M, Iacopino A and Christakos S (1990) Specific reduction of calcium-binding protein gene expression in aging and neurodegenerative diseases. *PNAS USA* 87:4078-4082.

Baimbridge K, Miller J and Parkes C (1982) Calcium-binding protein in the rat brain. *Brain Res* 239:519-525.

Baimbridge KG, Celio MR and Rogers JH (1992) Calcium-binding proteins in the nervous system. *Trends Neurosci* 15: 303-308.

Fearnley JM and Lees AJ (1991) Ageing and parkinson's disease: substantia nigra regional selectivity. Brain 114:2283-2301.

German DC, Manaye KE, Sonsalla PK and Brooks BA (1992) Midbrain dopaminergic cell loss in Parkinson's disease and MPTP-induced parkinsonism: sparing of calbindin-D_{28k} containing cells. *Ann NY Acad Sci* 648:42-62.

Heizmann C and Braun K (1992) Changes in Ca^{2+}-binding proteins in human neurodegenerative disorders. *Trends Neurosci* 15(7):259-264.

Iacopino A, Christakos S, German DC, Sonsalla PK and Altar CA (1992) Calbindin D_{28k}-containing neurons in animal models of neurodegeneration. *Mol Brain Res* 13:251-261.

Jande SS, Tolnai S and Lawson DEM (1981) Immunohistochemical localization of vitamin D-dependent calcium-binding protein in duodenum, kidney, uterus and cerebellum of chickens. *Histochemistry* 71:99-116.

Jenner P and Olanow CW (1996) Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* 47(Suppl 3): S161-S170.

Jenner P, Schapira AHV and Marsden CD (1992) New insights into the cause of Parkinson's disease. *Neurology* 42:2241-2250.

Koller WC (1997) Neuroprotective therapy for Parkinson's disease. Exp Neurol 144(1):24-28.

Lavoy B and Parent A (1991) Dopaminergic neurons expressing calbindin in normal and parkinsonian monkeys. *Neuroreport* 2:601-604.

Lee CS, Sauer H and Bjorklund A (1996) Dopaminergic neuronal degeneration and motor impairments following axon terminal lesion by intrastriatal 6-hydroxydopamine in the rat. *Neuroscience* 72(3):641-653.

Lindsay RM, Altar CA, Cedarbaum JM, Hyman C and Wiegand SJ (1993) The therapeutic potential of neurotrophic factors in the treatment of Parkinson's disease. *Exp Neurol* 124:103-118.

Parkinson Study Group (1993) Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease.

NEJM 328:176-183.

Paxinos G and Watson C (1986) The rat brain in stereotaxic coordinated. *Academic Press Australia*

Perese DA, Ulman J, Viola J and Ewing SE (1989) A 6-hydroxydopamine-induced selective parkinsonian rat model. *Brain Research* 494:285-293.

Rogers JH (1992) Immunohistochemical markers in rat brain: colocalization of calretinin and calbindin-D_{28k} with tyrosine hydroxylase. *Brain Res* 587:203-210.

Sauer H and Oertel WH (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immynocytochemical study in the rat. *Neuroscience* 59(2):401-415.

Yamada T, McGeer PL, Baimbridge KG and McGeer EG (1990) Relative sparing in Parkinson's disease of substantia nigra dopaminergic neurons containing calbindin-D_{28k}. *Brain Res* 526:303-307.