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Rapid Communication

Rescue of Dying Neurons: A New Action for Deprenyl in MPTP Parkinsonism

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Deprenyl slows the progression of disabling symptoms in Parkinson's disease (PD) by an unknown mechanism. It can block the action of MPTP on substantia nigra compacta (SNc) neurons by inhibiting monoamine oxidase B necessary to mediate the conversion of MPTP to MPP+, its active metabolite, in astroglia. Mice were pretreated with saline or the PD-producing toxin, MPTP (30 mg/kg) daily for 5 days and then after a further 3 days (to allow for the metabolism and excretion of the MPTP) were treated with deprenyl (0.25 or 10 mg/kg) or saline 3 times weekly for 20 days. In three series of mice treated with MPTP alone or MPTP-saline, serial sections through the SNc showed that averages of 37–42% of tyrosine hydroxylase (TH) immunoreactive neurons were lost gradually over 20 days. Joint counts of the numbers of TH-immunoreactive and Nissl-stained SNc somata from immediately adjacent sections established that the reductions in the numbers of TH-immunoreactive somata at 20 days after MPTP treatment represented neuronal death. Deprenyl treatment reduced the loss of TH-immunoreactive SNc neurons to averages of 14–16% for the 10-mg/kg and 0.25-mg/kg doses, respectively, and joint Nissl/TH counts for adjacent sections showed that reduction in the loss of TH-immunoreactive soma represented the rescue of SNc neurons that would have died by 20 days. The gradual loss of SNc neurons over the 20 days following MPTP exposure may reflect the toxin's axotomy-like effects on SNc neurons or the prolonged action of sequestered MPP+. In either case, the research shows that deprenyl can increase SNc neuronal survival by a mechanism that is independent of the blockade of MPTP's conversion to MPP+ and may be responsible for slowing the progression of PD.

Key words: deprenyl, substantia nigra, tyrosine hydroxylase, immunocytochemistry

INTRODUCTION

Deprenyl was originally used in combination with levodopa in the treatment of Parkinson's disease (PD) based on the hypothesis that it facilitates dopaminergic neurotransmission through its action as a monoamine oxidase B (MAO-B) inhibitor (Birkmayer et al., 1975). It is now used without additional levodopa therapy following the reports of the DATATOP project (Parkinson, 1989; U.S.A., 1989) and an independent study (Tetrud and Langston, 1989), which found that deprenyl delayed the onset of disabling symptoms requiring levodopa therapy by nearly 1 year. Although the design and conclusions of the DATATOP study have been controversial (Landau, 1990), the results of the two studies raise questions as to how deprenyl might slow the progression of PD.

Viewed simply, deprenyl might improve the function of surviving dopaminergic neurons in the compact part of the substantia nigra (SNc) (Knoll, 1988), or it might slow the death of dopaminergic SNc neurons (Langston, 1988), which is the major pathological feature of PD (Forno, 1982). MAO-B inhibitors like deprenyl can block 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism by preventing the conversion of MPTP to its toxic metabolite, MPP+ by MAO-B (Heikkila et al., 1984; Langston et al., 1984) in astroglia (Ransom et al., 1987; Takada et al., 1990). In a similar manner, deprenyl might minimize the generation of hydrogen peroxide associated with dopamine catabolism through its action as an MAO-B inhibitor (Cohen and Spina, 1989) and thereby slow the progression of PD by minimizing free-radical induced death of dopaminergic SNc neurons (Langston, 1988). However,

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reduction of free radical production by inhibition of MAO-B is not likely to occur within mammalian dopaminergic SNc neurons, since they do not contain MAO-B (Pintari et al., 1983; Vincent, 1989; Westlund et al., 1985, 1988). Hence deprenyl may slow the progression of PD by a mechanism other than its blockade of toxic conversions like the one found for MPTP (Ransom et al., 1987). It is not known whether deprenyl can influence SNc neuronal survival independent of the conversion of MPTP or MPP+. Mice offer an opportunity to investigate the action of deprenyl on dying SNc neurons, since the metabolism, excretion, and conversion of MPTP are markedly accelerated compared with primates (Johannessen et al., 1985; Lau et al., 1988; Markey et al., 1984). The action of deprenyl can therefore be investigated after the conversion of MPTP to MPP+ is completed (Song-cheng et al., 1988).

METHODS

In the first part of the study, MPTP (30 mg/kg/day) was administered intraperitoneally to 8-week-old isogenic C57BL mice (n = 6 per time period) for 5 consecutive days (total cumulative dose of 150 mg/kg). The mice were killed by anesthetic overdose followed by perfusion with isotonic saline (containing 5% rheomacrodex and 0.008% xylocaine) and 4% paraformaldehyde at 5, 10, 15, 20, 37, or 60 days following their last MPTP injection. In the second part of the study, saline or MPTP (30 mg/kg/day) was also administered intraperitoneally to 8-week-old mice (n = 6 per treatment group) for 5 consecutive days (days −5–0). Seventy-two hours following cessation of MPTP administration (day 0), the mice were treated with saline or deprenyl (0.25 or 10 mg/kg mg/kg i.p.) three times per week (i.e., on days 3, 5, 7, 10, 12, 14, 17, and 19). Mice were killed by anesthetic overdose followed by paraformaldehyde perfusion 20 days following their last MPTP injection.

For both parts of the study, the brains were bisected longitudinally along the midline and the half-brains from saline-alone and MPTP-alone or MPTP-saline and MPTP-deprenyl treated animals were joined together using Tissue-Tek so that surface landmarks coincided. The "glued" brains were frozen in −70°C. Methylbutane and then 10-μm serial sections were cut through the entire longitudinal length of each SNc. Alternate sections were reacted with a polyclonal antibody for tyrosine hydroxylase (TH, Eugene Tech.), then incubated with biotinylated secondary antibody, followed by incubation with HRP-conjugated avidin and finally reacted with diaminobenzidine and H_2O_2. TH-immunoreactive somata containing nuclear profiles were counted from the alternate sections taken serially through entire SNc nuclei and the values were corrected for counting from the alternate 10-μm sections (Konigsmark, 1970; Seniuk et al., 1990). Intervening sections were Nissl-stained to define nuclear outlines (see (Greenwood et al., 1991; Seniuk et al., 1990; Tatton et al., 1990) for more technical details). The glued sections for the paired half-brains ensured that any differences in neuronal numbers in the experimental and control groups were not due to different penetration or exposure to the antibodies or the reagents.

On 20 randomly chosen half-sections through the length of each nucleus for each animal, the region containing TH+ somata was outlined using a camera lucida attachment to the microscope and the outline was then transposed to the immediately adjacent Nissl section using local histological features for landmarks (each nucleus usually included about 90 pairs of sections). The numbers of Nissl somata containing a nucleolus were counted within the outline according to three size groups (small: 140–280 μm^2, medium: 300–540 μm^2, and large: 540–840 μm^2) and excluding glial profiles (40 to 100 μm^2) using criteria similar to those for the rat SNc (Poirier et al., 1983). Numbers of TH+ somata were plotted against numbers of Nissl somata for the corresponding areas of 20 immediately adjacent sections. The joint Nissl/TH+ counts provide a means of determining whether reductions in the numbers of TH+ SNc somata are due to neuronal destruction or to a loss of TH immunoreactivity by surviving neurons (see Seniuk et al., 1990; Tatton et al., 1991, for details as to rationale for the procedure).

RESULTS

We previously showed that MPTP produces a dose-dependent loss of TH+ SNc neurons by 20 days after administration of the toxin, so that doses of 30 mg/kg for 5 consecutive days caused the loss of 40–50% of the SNc dopaminergic neurons in C57 mice treated at 8 weeks of age (Seniuk et al., 1990). In the first part of the present study, we found that the loss of TH+ SNc somata occurs gradually over a 15 to 20-day period following completion of the 5-day course of MPTP administration, so that the numbers of TH+ somata do not reach their minimum value until 15 days after the completion of the MPTP treatment. These values then remain at an almost constant level at 20, 37, and 60 days (Fig. 1).

Joint plots of the counts of TH+ and Nissl-stained SNc somata from corresponding areas of immediately adjacent sections in mice treated with saline only (values for three animals are pooled in Fig. 2A1–A3) show that the numbers of TH+ somata are linearly related to the number of Nissl somata and are closely scattered around a one to one relationship (illustrated by the diagonal line in Fig. 2) for the medium-sized SNc somata (Fig. 2A2) and for the large-sized SNc somata (Fig. 2A3). In each

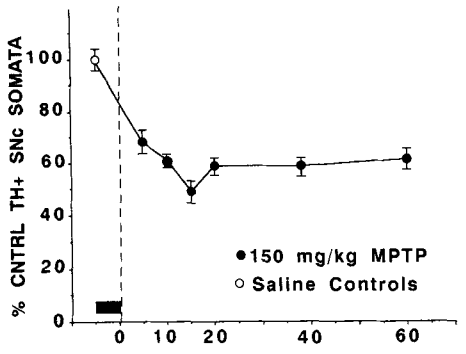

Fig. 1. Time course of the loss of tyrosine hydroxylase immunopositive (TH+) neurons from the substantia nigra comapcta (SNc) following MPTP administration. MPTP (30 mg/kg/day was administered over days −5 to −1. Note the gradual loss of TH+ SNc somata from days 5 to 15 or 20 days post MPTP which clearly began prior to our initial measurements at day 5. There is no appreciable loss after day 20.

plot in Figure 2, the mean ±1 SD for the Nissl counts and the TH+ counts of somata per half section are shown at the upper end of each y-axis and the right end of each x-axis, respectively. For the medium and large somata the mean number of Nissl somata exceed the corresponding mean number of TH+ somata by 5–10% which appears to correspond to the percentage of nigrostriatal neurons that are not TH+ (Van der Kooy et al., 1981). Joint counts of the small-sized SNc somata in the saline treated animals show that only a small proportion of the small neurons are TH-immunoreactive (Fig. 2A1). These results are consistent with previous findings in rodents, which indicated that the large- and medium-sized somata are dopaminergic nigrostriatal neurons, while the smaller somata are largely those of locally ramifying interneurons (Poirier et al., 1983; Van der Kooy et al., 1981).

Joint Nissl/TH counts of somata in the animals treated with MPTP alone or MPTP followed by saline (values for three MPTP-saline animals were pooled for presentation in Fig. 2B1, B2, B3) confirmed our previous finding that by 20 days after the completion of the MPTP treatment the loss of TH+ somata represented the death of SNc neurons rather than a loss of TH immunoreactivity in surviving neurons. Figure 2B2, B3 shows that even though the counts of Nissl and TH+ somata are reduced from 21.6 ± 15.5 and 20.6 ± 15.5 per section to 12.4 ± 8.0 and 11.4 ± 7.2 for the medium- and large-sized somata, respectively (values are means ±1.0 SD), the almost 1:1 relationships between the counts were maintained. If the SNc neurons were losing TH immunoreactivity but not dying, the scatter of the joint plots would be expected to shift to loci above the equal-value diagonal. Furthermore, Figure 2B1 shows

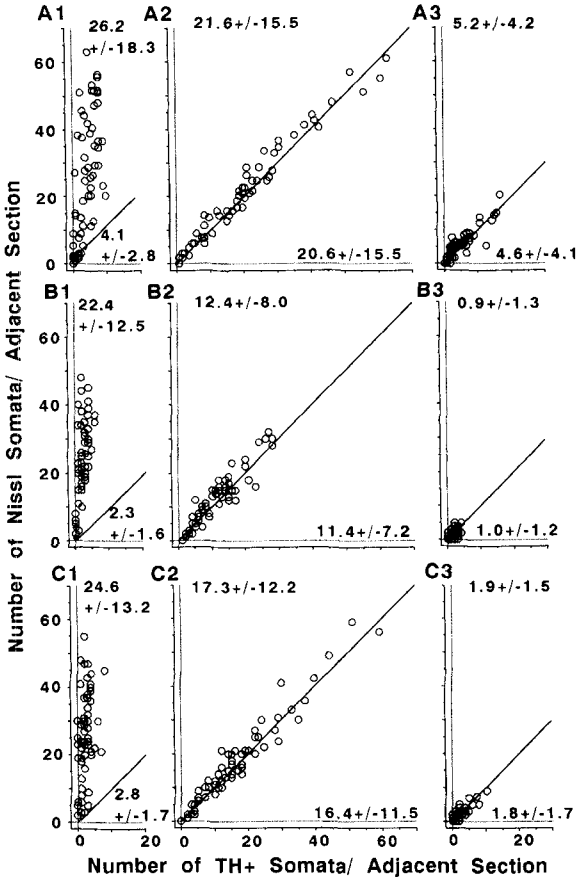

Fig. 2. Cumulative counts of TH+ somata along the rostrocaudal axis of the SNc nucleus. Note the cumulative raw counts of TH+ SNc somata vs. section number for individual representative SNc nuclei taken from alternate 10-μm serial sections throughout the entire nucleus. The pattern of the cumulative distribution curves for all the nuclei indicates that the loss of TH+ somata following MPTP occurs throughout the SNc nucleus. The degree of "rescue" of dying TH+ SNc neurons by deprenyl is such that there is no overlap in cumulative plots between the MPTP-Saline and the MPTP-Deprenyl animals.

that the numbers of small-sized Nissl-stained somata decreased slightly (26.2 ± 18.3 to 22.4 ± 12.5 per section), in accord with the reduction (4.1 ± 2.8 to 2.3 ± 1.6 per section) in the TH+ component of the small-sized SNc somata. If some of the medium- and large-sized SNc somata were atrophying, so that their cross-sectional areas no longer fell within the medium- and large-sized ranges in response to the MPTP treatment, one would expect an increase in the numbers of small-sized Nissl-stained somata.

Figure 3 presents three representative sets of four cumulative counts of TH+ SNc somata for individual SNc nuclei for alternate serial sections through the entire

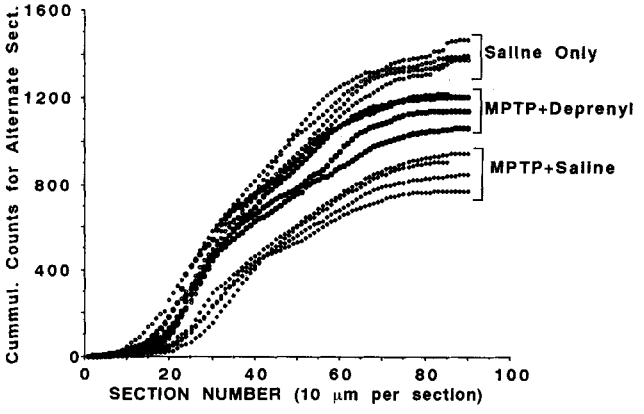

Fig. 3. Mean and SEM values for the MPTP, MPTP-saline, and MPTP-Deprenyl-treated mice. Note that all MPTP-treated mice had comparable levels of SNc neuronal loss, showing that saline injection had no effect and that deprenyl increased the number of TH+ SNc somata by about 60% relative to animals receiving MPTP alone or MPTP followed by saline. The two doses of deprenyl were equipotent in preventing the TH+ SNc neuronal loss.

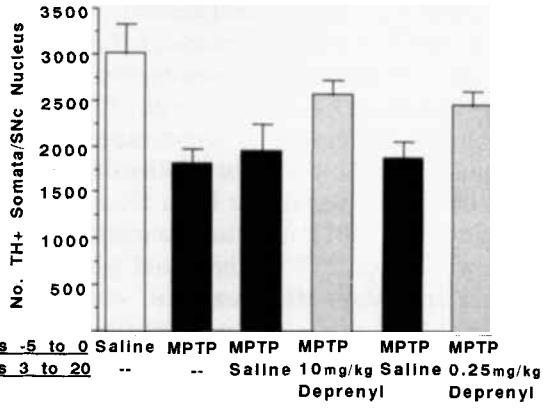

Fig. 4. Mean and SEM values for the MPTP, MPTP-Saline and MPTP-Deprenyl treated mice. Note that all MPTP-treated mice (MPTP and two groups of MPTP-Saline as black bars) had comparable levels of SNc neuronal loss, showing that saline injection had no effect on the MPTP induced loss of TH+ somata and deprenyl increased the number of TH+ SNc somata (grey bars) relative to animals receiving MPTP alone or MPTP followed by saline by about 60% showing that deprenyl prevented a major portion of the neuronal loss associated with MPTP-induced damage. Both doses of deprenyl were equipotent in preventing the TH+ SNc neuronal loss.

rostrocaudal length of each nucleus. The respective sets of cumulative counts are for animals treated with saline only, MPTP followed by deprenyl (MPTP+deprenyl) and MPTP followed by saline (MPTP+saline). The cumulative counts show that TH+ neuronal loss following MPTP occurred across the rostrocaudal length of the SNc when the saline-only and the MPTP-saline sets are compared. The cumulative counts for the MPTP-deprenyl set are considerably greater than the MPTP-saline set and do not overlap. Hence the "rescue" of dying neurons by deprenyl occurred in all parts of the nucleus, although it appears to be greatest in the rostral portion of the nucleus (sections 10–40).

Figure 4 shows that the mean corrected numbers of TH+ somata found for animals treated with saline only of 3,014 ± 304 (mean ±SEM) were significantly reduced (Mann–Whitney U-test, $P<.001$) in the animals treated with MPTP alone (1,756 ± 161) and in the two MPTP-saline groups (1,872 ± 187 and 1,904 ± 308). Therefore, MPTP caused average losses of 36%, 38%, and 42% of TH+ somata in the three MPTP-treated groups (black bars in Fig. 4). Deprenyl significantly increased ($P<.005$) the number of TH+ SNc somata after MPTP to 2,586 ± 161 (14% loss) and 2,535 ± 169 (16% loss) for the 10- and 0.25-mg/kg doses, respectively. Hence both doses of deprenyl reduced the loss of TH+ somata caused by the MPTP to less than 50% of the loss that was found when the MPTP was followed by saline.

The joint Nissl/TH+ counts in Figure 2C1–C3 were plotted for pooled data from three animals treated with MPTP followed by 0.25-mg/kg doses of deprenyl. Figure 2C2 shows a joint increase in the number of Nissl and TH+ medium-sized SNc somata compared to Figure 2B2 for the MPTP-saline animals. There is a relatively smaller increase in the joint number of large-sized somata for the MPTP-deprenyl animals (Fig. 2C3) compared with that for the MPTP-saline animals (Fig. 2B3). The joint Nissl/TH+ plots show that reduced loss of TH+ SNc somata in the MPTP-deprenyl treated mice is due to reduction in neuronal death rather than a reduction in the number of neurons that are not TH-immunoreactive.

DISCUSSION

These are the first data to indicate that deprenyl can alter the loss of SNc neurons caused by exposure to MPTP by a mechanism other than the blockade of MPTP conversion to MPP+. The observations may represent part of the mechanism that underlies the slowing of the progression of PD found for patients treated with deprenyl (Parkinson, 1989; Tetrud and Langston, 1989; USA, 1989). In previous experiments, MPTP and deprenyl were coadministered to monkeys or mice (Cohen et al., 1984; Del Zompo et al., 1986; Heikkila et al., 1984; Langston et al., 1984; Sheng et al., 1988), so that there was inhibition of MAO-B activity sufficient to prevent MPTP oxidation to MPP+ in astroglial cells (Ransom et al., 1987a,b). Studies using ^{3}H-MPTP have shown a

marked difference in liver metabolism and urinary excretion of MPTP in mice and monkeys (Lau et al., 1988) and the rates of loss of radioactivity from the brains of monkeys and mice (Johannessen et al., 1984). Both species show a rate of loss that is almost exponential, so that logarithmic plots show a linear relationship between the amount of label remaining in brain tissue and the time after a single ^{3}H-MPTP injection. In monkeys the loss is gradual with about 25% of the initial labeling of brain remaining at 20 days after treatment, while mice retain less than 15% of the initial brain labeling at 4 hr after treatment. Radiochemical HPLC detection has shown that 4 hr after administration of ^{14}C-labeled methyl-MPTP to mice, remaining label represents MPP+ and a more polar minor metabolite without any remaining MPTP (Song-cheng et al., 1988). Hence in our experiments, all of the conversion of MPTP to MPP+ was completed before the animals were started on deprenyl. The increased survival of SNc neurons at 20 days after MPTP was not mediated by a blockade of the conversion of MPTP to MPP+. The results indicate that deprenyl has a previously unidentified mechanism of action. Given the slow excretion and metabolism of MPTP in monkeys, that species cannot be used for experiments similar to those we have carried out in mice. It is possible that the reduction in TH+ neuronal death reported above might represent a delaying of neuronal death. Even if this is subsequently shown to be the case, however, the magnitude of the effect is striking and further long-term studies in mice will be needed to determine whether the increased number of surviving neurons at 20 days remain at longer intervals, such as 40 and 60 days after MPTP. It will also be interesting to learn whether longer-term survival of "rescued" neurons requires continued administration of deprenyl.

Both dosages of deprenyl were equipotent in rescuing the neurons, and it will be necessary to do direct measurements of MAO-B inhibition in mice to determine if MAO-B inhibition is a requirement for the rescue. In rats, a 0.25-mg/kg dose of deprenyl inhibits MAO-B by 50–75% and has been reported to prolong their lifespan (Demarest and Azzaro, 1979; Knoll, 1988a,b), while the 10-mg/kg dose inhibits most MAO-B and some MAO-A activity (Demarest and Azzaro, 1979). If similar inhibition occurs in mice, both doses that we used may act through MAO-B blockade. Similar experiments with a variety of doses and agents with differing capacities for MAO-B inhibition will be required to determine whether the rescue is dependent on MAO-B blockade.

If the rescue mechanism is shown to require inhibition of MAO-B, the mechanism is unlikely to be the result of a direct effect of deprenyl on dopaminergic neurons themselves because MAO-B is not found in SNc neurons (Pintari et al., 1983; Vincent, 1989; Westlund et al., 1985, 1988), leading to the speculation that glial cells might be playing a role in the rescue. Apart from an inhibition of glial MAO-B, various mechanisms may be operative, including reduced oxidative stress (Cohen and Spina, 1989), altered dopaminergic neurotransmission (Knoll, 1988a,b; Paterson et al., 1990) or an amelioration of the effect of MPP+ remaining within the SNc neurons (Campbell et al., 1990).

The decrease in the numbers of SNc neurons that die after MPTP exposure in animals treated with deprenyl is even more striking when one considers that 70–75% of the TH+ SNc neurons that would die by day 20 had already lost their TH-immunoreactivity by day 5 (Fig. 1). By simple linear interpolation of numbers derived from the saline controls and the MPTP-treated animals at 5 days, the numbers of TH+ SNc somata would have decreased from a mean of 3,014 somata/nucleus to about 2,169 at day 3 and then further declined to an average of 1,872 somata/nucleus by day 20. Deprenyl-treated mice had an average of 2535 somata/nucleus at day 20, thereby suggesting that deprenyl may have rescued almost all TH+ SNc neurons that had not died by the onset of deprenyl treatment including some of the SNc neurons that had lost their TH immunoreactivity before the onset of deprenyl treatment. Our mean value of 3014 TH+ neurons per SNc nucleus in animals treated with saline only is almost identical to that previously reported for the same mouse strain at a similar age (Gupta et al., 1990).

After conversion from MPTP in astroglia, MPP+ is taken up into dopaminergic terminals in the striatum (Javitch et al., 1985). MPP+ concentrates in mitochondria (Ramsay et al., 1986; Ramsay and Singer, 1986) in the terminals and blocks NAD-substrate oxidation (Nicklas et al., 1985; Ramsay et al., 1986, 1987a,b), causing destruction of the terminals within 24 hr of MPTP exposure (Linder et al., 1987) by the inhibition of mitochondrial respiration and ATP synthesis (Mizuno et al., 1987a,b; 1988). Kitt and her coworkers (Kitt et al., 1987) proposed that the decrease in TH immunoreactivity in SNc neurons that was evident 48 hr after MPTP exposure represented a reaction of the neurons to axon damage (Barron, 1986). We proposed that the death of the SNc neurons may be in some part due to the axonal damage produced by MPP+ (Seniuk et al., 1990; Tatton et al., 1990). The gradual death of the neurons over 15 days after MPTP exposure shown in the present experiments could also be the result of axonal damage. It has recently been shown that embryonic trophic factors or gangliosides can rescue MPTP-damaged SNc neurons over a time course similar to the action of deprenyl reported here (Date et al., 1990; Gupta et al., 1990; Hadjiconstantinou et al., 1986). Some neurontrophic factors have been shown to reduce the death of motoneurons

after section of their peripheral axons (Sendtner et al., 1990). It will be interesting to determine whether deprenyl can act similarly to reduce motoneuronal death after axonal section which could indicate a neuronotrophic-like action as part of the rescue of dying SNc neurons.

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