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Lazaroids improve the survival of grafted rat embryonic dopamine neurons

(neural transplantation/21-aminosteroids/Parkinson disease/free radicals/behavioral recovery)

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ABSTRACT **In rodent models of Parkinson disease in which transplants of dissociated rodent and human embryonic mesencephalic tissue, rich in dopamine neurons, have been studied, only 5–20% of the dopamine neurons survive the implantation procedure. We have investigated the effects of inhibiting free radical generation with two lazaroids, U-74389G and U-83836E, on the survival of embryonic rat dopamine neurons. U-74389G is a 21-aminosteroid, and U-83836E combines the piperazinyl pyrimidine portion of 21-aminosteroids with the antioxidant ring of α-tocopherol. In an initial study, we found that the lazaroids markedly prolonged the period after tissue dissociation that an embryonic mesencephalic cell suspension exhibits high cell viability *in vitro*, as assessed by using a dye exclusion method. In a second series of experiments, addition of lazaroids to dissociated mesencephalic graft tissue increased the yield of surviving rat dopamine neurons 2.6-fold after implantation in the dopamine-denervated rat striatum. The improved survival correlated with an earlier onset of graft-induced functional effects in the amphetamine-induced rotation test. Thus, inhibition of free radical generation can significantly increase the yield of grafted embryonic dopamine neurons. Addition of lazaroids to the graft preparation is a relatively simple modification of the transplantation protocol and could readily be applied in a clinical setting. Moreover, since iron-dependent lipid peroxidation has been suggested to play a role in the death of nigral dopamine neurons in Parkinson disease and lazaroids are particularly potent inhibitors of such processes, the findings may have implications for the pathogenesis of this disease.**

Several studies indicate that grafts of human embryonic dopamine neurons can survive and reduce motor symptoms after transplantation to the brains of patients with Parkinson disease (1). However, the results obtained so far indicate that the symptomatic relief is far from complete and it has been suggested that improved functional effects would be attainable if the grafts contained more surviving dopamine neurons and innervated a larger volume of the denervated parkinsonian striatum (1). Experiments with transplants of rat and human mesencephalic dopamine neurons placed in the rat striatum have shown that on the order of 5–20% of the dopamine neurons that are dissected from the embryos survive the stereotaxic implantation procedure when a protocol similar to that employed clinically is used (2–5). The underlying reason for the relatively poor survival rate of grafted dopamine neurons is not known. However, we have hypothesized (6) that the major loss of dopamine neurons occurs either during dissection and mechanical dissociation of the graft tissue or soon after implantation into the adult brain environment.

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It has been suggested that the generation of oxygen free radicals and consequent lipid peroxidation of cell membranes are involved in the pathogenesis of neuronal injury in cerebral ischemia (7, 8), trauma (7, 8), and neurodegenerative disorders (9, 10). Indeed, it has been hypothesized that dopamine neurons are particularly susceptible to oxidative stress and that free radicals may play a role in the pathogenesis of Parkinson disease (9). Thus, our working hypothesis is that the inevitable cellular hypoxia and trauma that occur during preparation and grafting of the embryonic mesencephalic tissue lead to the production of oxygen free radicals, which provoke membrane lipid peroxidation, consequently reducing the survival of dopamine neurons in the transplants.

Recently, a group of compounds, the lazaroids, have been developed for acute treatment of central nervous system injury (11). They are 21-aminosteroids that lack glucocorticoid activity and are specifically designed to localize within cell membranes and inhibit lipid peroxidation (11). Lazaroids have been shown to ameliorate tissue damage in animal models of central nervous system trauma, ischemia, and subarachnoid hemorrhage (11, 12). Clinical trials are underway testing lazaroids in patients with acute ischemic stroke (13).

The present study was designed to evaluate the effects of two lazaroids, U-74389G and U-83836E, on the survival of embryonic mesencephalic rat dopamine neurons. U-74389G is a 21-aminosteroid, whereas U-83836E combines the piperazinyl pyrimidine antioxidant portion of 21-aminosteroids with the antioxidant ring of α-tocopherol. In the first part of the study, the influence of lazaroids on the cell viability in a preparation of dissociated mesencephalic embryonic cells was monitored *in vitro* by using a dye exclusion method. In the second part of the study, the survival of embryonic rat dopamine neurons grafted to the striatum was assessed behaviorally and morphologically in a rat model of Parkinson disease.

MATERIALS AND METHODS

Unilateral 6-Hydroxydopamine Lesion and Motor Asymmetry Test. Female Sprague–Dawley rats were subjected to unilateral 6-hydroxydopamine lesions of the ascending mesostriatal dopamine pathway as described (5). The effect of the 6-hydroxydopamine lesion was assessed by monitoring amphetamine (2.5 mg/kg, i.p.)-induced turning behavior (14). The rats that exhibited a net rotational asymmetry (turns contralateral to the lesion subtracted from ipsilateral turns) of at least six full turns per min toward the lesioned side, which is consistent with an average of a 99% striatal dopamine depletion (15), were selected for transplantation surgery. The rotation tests were repeated at 2 and 6 weeks after transplantation surgery.

Abbreviation: TH, tyrosine hydroxylase.
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Preparation of Cell Suspensions. Cell suspensions for both *in vitro* viability testing and intracerebral transplantation were prepared from ventral mesencephalic tissue obtained from embryonic day 14 rat embryos (Sprague–Dawley; crown-to-rump length, 12–13 mm) as described (5). In the transplantation experiment, two pregnant rats were injected (10 mg/kg, i.p.) with either U-74389G (dissolved in 0.05 M HCl) or U-83836E (dissolved in saline) 1 hr prior to removal of the embryos. These pregnant rats supplied the embryos for the U-74389G and U-83836E groups, respectively. Embryos for the control transplantation group and all the groups in the *in vitro* cell viability experiments were taken from untreated pregnant rats. The ventral mesencephalic tissue was dissected under aseptic conditions in Hanks' balanced salt solution (HBSS) and the tissue pieces were incubated in 0.1% trypsin (Worthington)/0.05% DNase (Sigma)/HBSS at 37°C for 20 min. After rinsing four or five times with 0.05% DNase/HBSS, the tissue was dissociated by trituration through the tip of a fire-polished Pasteur pipette into a single-cell suspension, almost free from aggregates. For each dissected ventral mesencephalon, 10 μl of the dissociation medium was added. In the lazaroid-treated groups, either U-74389G or U-83836E (Upjohn) was added to a concentration of 0.3 μM to the medium at each step of the procedure, including the tissue dissection, incubation, and dissociation. U-74389G was first dissolved in dimethyl sulfoxide, followed by dilution with 3% (wt/vol) fatty-acid-free bovine serum albumin/HBSS because of its limited solubility in electrolyte solutions at neutral pH. This solution was further diluted with HBSS to working concentrations. U-83836E was dissolved in HBSS to be diluted to the working concentration of 0.3 μM. In all cases, special care was taken to balance the number of embryos and the volume of dissociation medium for each group to obtain cell suspensions with a similar cell concentration.

***In Vitro* Cell Viability Assessment.** The cell suspensions were maintained at ambient room temperature (20–22°C) for up to 96 hr. At set intervals, 1 μl of each cell suspension was withdrawn and mixed with 9 μl of a mixture of acridine orange (Sigma) and ethidium bromide (Sigma; 3.4 μg/ml of each component in 0.9% NaCl) (16). The stained suspension was transferred to a hemocytometer, and an average of 400 viable and nonviable cells were counted using a fluorescence microscope under epiillumination at 390 nm. Cell viability was monitored starting 1 hr after the dissociation step and at defined 1- to 12-hr intervals until the percent viability fell below 30%.

Transplantation Surgery. Recipient rats were divided into three groups: (*i*) control (n = 7), (*ii*) U-74389G-treated (n = 7), and (*iii*) U-83836E-treated (n = 7) groups. The cell viability of the cell suspensions just prior to grafting was 92–94% in all groups. The cell concentrations in the control, U-74389G-treated, and U-83836E-treated, groups were 4.07 × 10^4 cells per μl, 4.03 × 10^4 cells per μl, and 4.03 × 10^4 cells per μl, respectively. Under equithesin anesthesia, two stereotaxic 2-μl deposits of the cell suspension (20% of one mesencephalon, containing 8 × 10^4 viable cells) were injected into the right (lesioned) striatum of recipient rats (5).

Morphological Assessment. Six weeks after graft surgery, the rats were deeply anesthetized with chloral hydrate and perfused transcardially with phosphate-buffered 4% (wt/vol) paraformaldehyde, and their brains were processed for tyrosine hydroxylase (TH) immunohistochemistry (Pel-Freez Biologicals; antibody dilution 1:500), as described (5).

The number of TH-immunoreactive neurons in each graft was counted on every third section on blind-coded slides, and the raw counts were corrected by using the formula of Abercrombie (17). Individual graft volumes were determined using a Macintosh-based computerized image processing system. Histological sections were digitized using a high-resolution charge-coupled device videocamera (Dage–MTI, Michigan City, IN) in a light box (Imaging Research, St. Catherine's, ON, Canada) and a software package, IMAGEGRABBER (Neotech, Hampshire, U.K.), and analyzed using IMAGE software (version 1.52, National Institutes of Health). The graft perimeter of each digitized image was outlined manually on the screen and the area within the borders was calculated and expressed in mm^2. Every third section was analyzed and the total graft volume was calculated by taking into consideration section thickness and frequency.

RESULTS

***In Vitro* Cell Viability Assessment.** The changes in cell viability over time for the three groups are shown in Fig. 1. In all groups the initial mean viability rate was >90%. In the control group, the cell viability showed a rapid decrease around 12–18 hr after tissue dissociation. In contrast, the viability level in both lazaroid-treated groups was maintained at ≈80% until 60 hr after dissociation, after which it rapidly decreased. Two-way analysis of variance (ANOVA) revealed a significant group–time interaction ($P < 0.0001$) when the three groups were analyzed, but there was no significant group–time interaction between the two lazaroid-treated groups when analyzed separately ($P > 0.05$), indicating that the decline in cell viability was significantly faster in the control than in the two lazaroid-treated groups.

Amphetamine-Induced Rotation. The amphetamine-induced rotation scores are summarized in Fig. 2. At 2 weeks after transplantation, the proportion of rats in each group that exhibited at least 50% reduction in net motor asymmetry compared to pretransplantation values was as follows: control group, 3 of 7; U-74389G-treated group, 7 of 7; U-83836E-treated group, 7 of 7. Six weeks after surgery, all the rats exhibited >50% reduction of net motor asymmetry.

Two-way ANOVA revealed a significant group–time interaction ($P < 0.05$) of mean net rotation asymmetries at the three time points, reflecting that the lazaroid-treated rats exhibited a more rapid behavioral recovery than the rats in the control group. Indeed, 2 weeks after grafting, the mean net rotation values were significantly reduced, compared to pregrafting values, in the U-74389G- and the U-83836E-treated groups ($P < 0.001$, paired Student's t test) but not in the control group ($P > 0.05$). One-way ANOVA with post-hoc Scheffé's tests showed that 2 weeks after grafting both lazaroid-treated groups exhibited lower net asymmetry values than the control group ($P < 0.02$), whereas scores for the two lazaroid-treated groups did not significantly differ ($P > 0.05$). At 6 weeks after surgery, the three groups did not differ

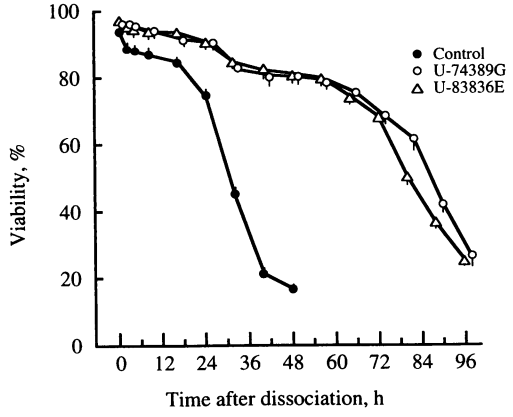

FIG. 1. Changes in viability *in vitro*, determined by using acridine orange/ethidium bromide dye exclusion, of mesencephalic cell suspensions over time after mechanical dissociation. Data are expressed as the mean ± SEM of four experiments.

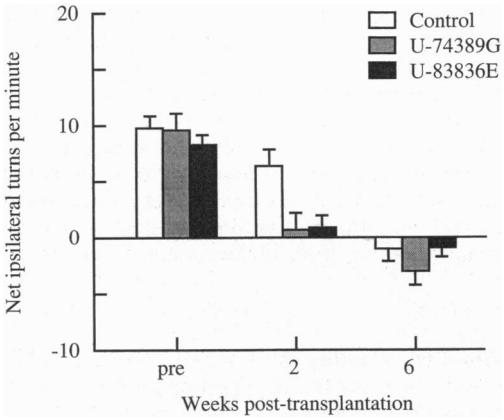

FIG. 2. Net ipsilateral amphetamine-induced rotation asymmetry (full turns per min contralateral to the lesion subtracted from turns ipsilateral to the lesion) over the 90-min test session for the control and the lazaroid-treated groups. The rats were tested before grafting and 2 and 6 weeks after grafting. Each bar represents the group mean and the error bars denote SEM.

from each other ($P > 0.05$), and all showed complete reversal of rotational asymmetry and displayed scores that were significantly reduced compared to pretransplantation values ($P < 0.0001$, paired Student's t test, within each group).

Graft Survival and Volume. Light microscopic analysis of TH-immunostained brain sections revealed two grafts, located in the lateral and medial striatum of all rats. TH-positive cell bodies were primarily located at the periphery of each graft (Fig. 3). There were no significant differences in either the numbers of TH-positive cells or graft volumes between the lateral and medial grafts ($P > 0.05$, paired Student's t test) in any group. The mean number of surviving TH-positive neurons in each experimental group is summarized in Fig. 4*A*. Treatment with U-74389G and U-83836E produced significant increases in the mean number of TH-positive neurons to 265% and 263%, respectively, of control ($P < 0.02$, one-way ANOVA with post-hoc Scheffé's test). No significant difference in the survival of TH-positive neurons was observed between the U-74389G- and the U-83836E-treated groups ($P > 0.05$). The grafts in the lazaroid-treated groups were significantly greater in volume than the control grafts ($P < 0.01$, one-way ANOVA with post-hoc Scheffé's test), whereas there was no significant difference in graft volume between the two lazaroid-treated groups ($P > 0.05$; Fig. 4*B*). The number of TH-positive cells

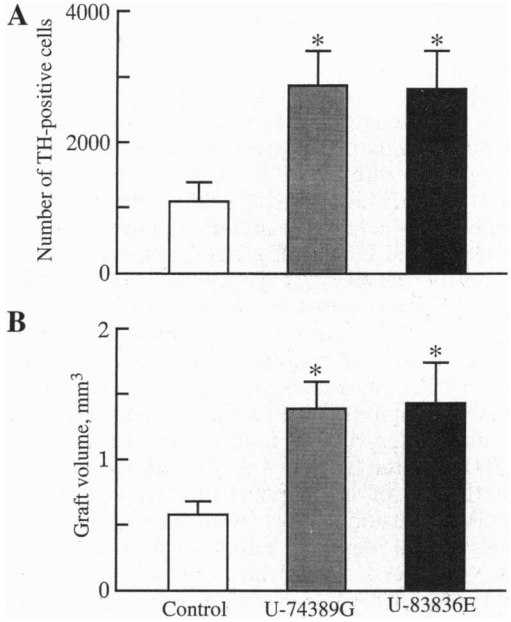

FIG. 4. Mean number of surviving TH-positive neurons (*A*) and the mean graft volume (*B*) for the control, the U-74389G-treated, and U-83836E-treated groups. * indicates $P < 0.01$ (one-way ANOVA with post-hoc Scheffé's test) compared to control group. Error bars indicate SEM.

in each graft and the graft volume were highly correlated ($r^2 = 0.746$; $P < 0.0001$, linear regression analysis with data from all three groups).

Relationship Between Graft Survival and Functional Effects. In Fig. 5*A*, the sum of TH-positive cells found in the two grafts in each rat is plotted against the percent reduction of pretransplantation scores in net rotational asymmetry 2 weeks after graft surgery. Logarithmic regression analysis revealed a significant correlation between the two parameters (2 weeks after grafting: $r^2 = 0.767$; $P < 0.0001$). When a similar analysis was performed on the behavioral data obtained 6 weeks after grafting, the correlation was less strong but still statistically significant ($r^2 = 0.421$; $P < 0.01$). From the curve obtained by logarithmic regression analysis, it can be deduced that it is necessary to have ≈2000 surviving TH-positive neurons in the grafts to induce a 50% reduction in motor asymmetry at 2 weeks after grafting. The number of TH neurons required for an equivalent behavioral effect to develop by 6 weeks after surgery is ≈400.

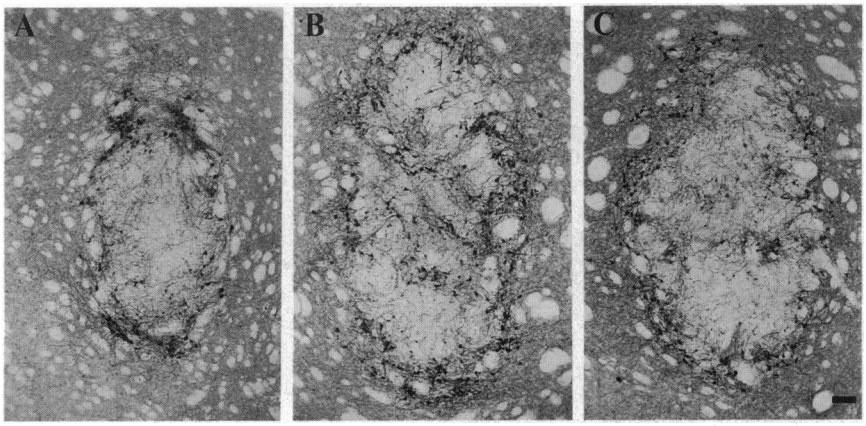

FIG. 3. Photomicrographs of coronal sections through the host striatum of one representative rat from each group processed for TH immunohistochemistry. Each photograph illustrates one out of the two grafts implanted in each rat. Control (*A*), U-74389G-treated (*B*), and U-83836E-treated (*C*) groups are shown. The total numbers of surviving TH-immunopositive neurons in the depicted grafts were as follows: *A*, 1281; *B*, 2765; *C*, 2452. (Bar = 100 μm.)

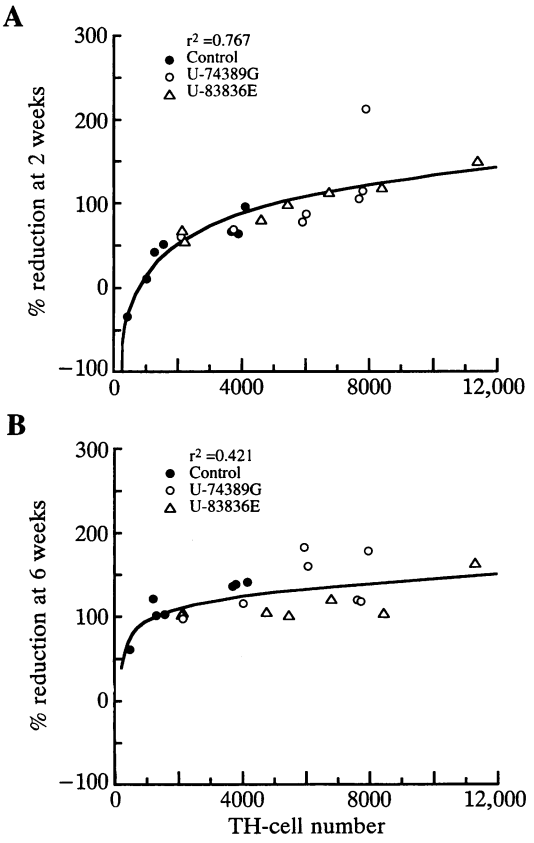

FIG. 5. Sum of the number of TH-positive cells in the two grafts in each rat is plotted against the individual percentage reduction in net rotational asymmetry 2 (*A*) and 6 (*B*) weeks after graft surgery. The plotted curves were obtained by logarithmic regression analysis (2 weeks after grafting, $r^2 = 0.767$ and $P < 0.0001$; 6 weeks after grafting, $r^2 = 0.421$ and $P < 0.01$). Each symbol represents one rat.

DISCUSSION

Addition of two lazaroids, U-74389G and U-83836E, to embryonic mesencephalic cell suspensions was found to increase the period of high cell viability *in vitro* and also increased the yield of surviving dopamine neurons 2.6-fold when the cells were transplanted to the striatum. In the cell viability experiment, the cell suspensions were maintained at room temperature for several hours after mechanical dissociation. The control suspensions exhibited a high viability up to 6 hr, before they showed a gradual decline in viability down to ≈20% after 24–30 hr. This drop in viability has been observed previously in mesencephalic cell suspensions prepared from embryonic day 14 or 15 rat embryos that 6–9 hr after preparation exhibited a rapid decline to viability rates of 20–40% (16). Viability assessed with the acridine orange/ethidium bromide dye-exclusion method, as utilized in the present study, presents an index of membrane integrity of the cells. The lazaroids used in this experiment are believed to intercalate into cell membranes by embedding their hydrophobic portion into the core of the lipid membrane and there potently inhibit lipid peroxidation, protecting the cellular membrane (11). It is believed that lazaroids also can protect membranes via a direct interaction with the lipid bilayer and by shielding membrane proteins from proteolytic attack by an antioxidant action (11, 18). In our experiment, the lazaroids maintained the cell viability in the mesencephalic cell suspensions at ≈80% for 60 hr after tissue dissociation. Earlier work has indicated that there is a clear correlation between the *in vitro* viability of mesencephalic cell suspensions and the number of dopamine neurons that survive grafting from the same tissue preparation (16, 19). Thus, manipulations that prolong the period of high cell viability for a cell suspension *in vitro* can be postulated to be beneficial to the survival of dopamine neurons after intracerebral implantation of the cell suspension.

There is a strong correlation between the survival of mesencephalic grafts in rats and their effects on the behavioral deficits that follow dopaminergic denervation of the striatum (2, 15, 16, 19–23). Therefore, it is feasible that improving the survival of dopamine neurons grafted to patients with Parkinson disease could increase the symptomatic relief beyond the significant effects on motor function that have been observed in clinical studies so far (1). Based on animal experiments, the survival rate of transplanted rat and mouse dopamine neurons is 10–20% (2, 3), and the survival rate for human dopamine neurons xenografted to the immunosuppressed rat is ≈5% (4, 5). There have been several attempts to improve the dopamine neuron yield by various modifications in the transplantation protocol. However, there has been no major beneficial effect on neuronal survival in mesencephalic implants by the addition of gangliosides (24, 25), brain-derived neurotrophic factor (26), nerve growth factor (26), or embryonic striatal target neurons (27, 28). Recently, Mayer *et al.* (29) observed a >100% increase in the number of dopamine neurons in mesencephalic grafts treated with basic fibroblast growth factor, either in the graft cell suspension or by repeated intracerebral infusions for 20 days. However, when the graft survival time exceeded 3 weeks, the beneficial effect was significant only in the rats with intracerebral infusions. These rats had marked inflammatory reactions around the sites of infusion, which complicates the interpretation of results and, as the authors point out (29), would make it difficult to use this approach clinically.

The results of the current study indicate that inhibition of lipid peroxidation by the treatment of donor tissue with lazaroids increases the survival of dopamine neurons 2.6 times in intrastriatal grafts. Previous estimations of survival rates of transplanted dopamine neurons have been based on the assumption that the rat mesencephalon contains 30,000–40,000 dopamine neurons (30). Since in the present study each graft deposit contained tissue equivalent to one-fifth of one embryonic mesencephalon, 6000–8000 dopamine neurons were allocated to each graft. Thus, the control grafts that contained a mean of ≈1100 surviving TH-immunopositive dopamine neurons displayed a dopamine neuron survival rate of 14–18%, which is in good agreement with previous work on grafted rat and mouse mesencephalic tissue (2, 3). The survival rate was markedly increased by the treatment with lazaroids and reached a level of 36–48% (≈2900 TH-positive neurons) for both the U-74389G- and the U-83836E-treated groups. The marked neuroprotective effect of the lazaroids on embryonic rat neurons warrants testing these compounds on human mesencephalic xenografts to rats, and in the event of a similar protective effect, the approach of inhibiting free radical processes may be tested in clinical transplantation trials. Since lazaroids are being tested as neuroprotective agents in stroke patients (13) and have undergone clinical safety testing (31), the logistics of such a clinical application are facilitated.

The correlation between the logarithm of the dopamine neuron cell number in the grafts in each rat and the degree of recovery in the rotation test was stronger at 2 weeks after surgery than at 6 weeks. This is probably due to almost all of the rats carrying implants of a size exceeding that necessary to reverse amphetamine-induced motor asymmetry 6 weeks after surgery. Previous studies (2, 21) have shown that after such a relatively long transplant survival time, only 400–600 TH-immunopositive neurons are required to induce a 50% reduction in amphetamine-induced rotational asymmetry. From the present data, it is clear that for a similar functional

effect to develop by 2 weeks after surgery, larger grafts containing as many as 2000 TH-immunopositive neurons are needed. Thus, when studying manipulations that may increase the number of surviving grafted dopamine neurons above a relatively high baseline number, it is useful to test for amphetamine-induced rotation as early as 2 weeks after transplantation.

The findings of neuroprotective effects of lazaroids in the present study are particularly important in the light of the fact that there is currently speculation that oxygen free radicals play a role in several types of brain and spinal cord damage, such as trauma, ischemia, and certain neurodegenerative disorders (7–10). Recently, it was found (32) that patients with familial amyotrophic lateral sclerosis exhibit a defect in a gene for superoxide dismutase, which acts as part of an endogenous free radical scavenger system. The adult substantia nigra is believed to be particularly vulnerable to oxidative stress since it contains high levels of free iron that can catalyze lipid peroxidation and is rich in dopamine that can readily undergo oxidative metabolism to form reactive quinones and hydrogen peroxide, which in turn can generate toxic hydroxyl radicals (9). Iron-dependent lipid peroxidation has in fact been suggested to play a role in the death of nigral dopamine neurons in Parkinson disease (9, 10, 33, 34) and since lazaroids are particularly potent inhibitors of such processes, the findings of the present study may even have implications for the pathogenesis of this disease.

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