

Improvement of embryonic dopaminergic neurone survival in culture and after grafting into the striatum of hemiparkinsonian rats by CEP-1347

Jette Bisgaard Boll,* Marie Aavang Geist,* Gabriele S. Kaminski Schierle,† Karina Petersen,‡, Marcel Leist* and Elisabetta Vaudano§

*Departments of Molecular Disease Biology, ‡Molecular Genetics and §Neurodegenerative Disorders, H. Lundbeck A/S, Valby, Denmark

†Cambridge Center for Brain Repair, University of Cambridge, Cambridge, UK

Abstract

Transplantation of embryonic nigral tissue ameliorates functional deficiencies in Parkinson's disease (PD). A main constraint of neural grafting is the poor survival of dopaminergic neurones grafted into patients. Studies in rats indicated that many grafted neurones die by apoptosis. CEP-1347 is a mixed-lineage-kinase (MLK) inhibitor with neuroprotective action in several *in vitro* and *in vivo* models of neuronal apoptosis. We studied the effect of CEP-1347 on the survival of embryonic rat dopaminergic neurones in culture, and after transplantation in hemiparkinsonian rats. CEP-1347 and the alternative MLK inhibitor CEP-11004 significantly increased the survival of dopaminergic neurones in primary cultures from rat ventral mesencephalon and in Mn²⁺-exposed PC12 cells, a

surrogate model of dopaminergic lethal stress. Moreover, combined treatment of the grafting cell suspension and the host animal with CEP-1347 significantly improved the long-term survival of rat dopaminergic neurones transplanted into the striatum of hemiparkinsonian rats. Also, the protective effect of CEP-1347 resulted in an increase in total graft size and in enhanced fibre outgrowth. Thus, treatment with CEP-1347 improved dopaminergic cell survival under severe stress and might be useful to improve the positive outcome of transplantation therapy in PD and reduce the amount of human tissue required.

Keywords: CEP-1347, dopaminergic, mixed-lineage-kinase, neuroprotection, substantia nigra, transplantation.

Transplantation of embryonic ventral mesencephalic tissue containing dopaminergic neurones is an effective treatment for Parkinson's disease (PD) (Kordower *et al.* 1998; Tabbal *et al.* 1998; Piccini *et al.* 1999; Brundin *et al.* 2000b; Lindvall and Hagell 2000; Freed *et al.* 2001). However, one of the major restrictions of more widespread application of clinical transplantation is the limited availability of suitable donor tissue, combined with a poor survival of dopaminergic cells in the grafts, i.e. a maximum of 5–10% in rats, monkeys and human (Brundin *et al.* 1985; Kordower *et al.* 1998; Zawada *et al.* 1998; Hagell and Brundin 2001). Around 20–30% of the mesencephalic cells already die during the preparation of donor tissue prior to transplantation (Fawcett *et al.* 1995; Kaminski Schierle *et al.* 1999a; Brundin *et al.* 2000a; Karlsson *et al.* 2000), probably due to traumatic and ischemic damage during dissection and dissociation

procedures. Another 67–70% of dopaminergic neurones die during the first week post transplantation (Barker *et al.* 1996; Emgard *et al.* 1999; Sortwell *et al.* 2000). A part of the cell death that accompanies embryonic neuronal transplantation of rat and human cells is apoptotic and associated with the activation of caspases (Branton and Clarke 1999; Schierle

et al. 1999; Brundin *et al.* 2000a). One method of increasing survival of dopaminergic neurones would be to prevent this form of cell death. Activation of the Jun N terminal kinase (JNK) stress pathway has been shown to be central to apoptotic neuronal cell death in several *in vitro* and *in vivo* models (Harper and LoGrasso 2001). More specifically, activation of JNK has been demonstrated in *in vitro* and *in vivo* models of dopaminergic neuronal death (Choi *et al.* 1999; Saporito *et al.* 2000; Chun *et al.* 2001; Gearan *et al.* 2001). CEP 1347 and CEP 11004 are mixed lineage kinase (MLK) inhibitors that have been found to be protective in a number of *in vitro* models and in an *in vivo* dopaminergic neuronal degeneration model (Maroney *et al.* 1998, 2001; Saporito *et al.* 1999; Bozyczko Coyne *et al.* 2001; Harris *et al.* 2002b; Murakata *et al.* 2002). Notably, these inhibitors do not inhibit JNK activity directly in cells but rather, act upstream by inhibiting the family of MLKs, a class of mitogen activated protein kinase kinases activated by stress and cytokines (Maroney *et al.* 2001). A critical role for MLK activation in JNK mediated neuronal apoptotic death has been demonstrated (Xu *et al.* 2001). Here we use the MLK inhibitor CEP 1347 to elucidate the role of MLK in the survival of embryonic dopaminergic neurones in culture and following transplantation into the denervated striatum of hemiparkinsonian rats.

Experimental procedures

Materials

Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS) without calcium and magnesium, HEPES, sodium pyruvate, penicillin/streptomycin, fetal bovine serum and horse serum were purchased from Gibco BRL (Invitrogen, Taastrup, Denmark). Trypsin was purchased from Worthington Biologicals Corporation (Medinova Scientific A/S, Hellerup, Denmark). Laza roid (U74389G), poly L lysine, DNase and all other chemicals were purchased from Sigma (Vallensbæk Strand, Denmark). Laboratory Tek chamber slides were purchased from Nunc A/S (Roskilde, Denmark). Hoechst 33342 was purchased at Molecular Probes (Leiden, the Netherlands). Mouse anti tyrosine hydroxylase (TH) monoclonal antibody was purchased from Chemicon (Chandlers Ford, UK). The 'c jun activation hit kit', was purchased from Cellomics (Pittsburgh, PA, USA). The p c jun and p JNK mouse monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal horse serum and biotinylated horse anti mouse IgG (H + L) were purchased from Vector Laboratories (VWR International, Albertslund, Denmark). Horseradish peroxidase conjugated anti mouse antibody, Avidin biotin peroxidase complex system [ABCComplex/horseradish peroxidase (HRP)] and 3,3' diaminobenzidine (DAB) chromogen were purchased from DAKO (Glostrup, Denmark). ECL Plus reagent was purchased from Amersham (Arlington Heights, IL, USA). JNK inhibitor I peptide and SP600125 were purchased from Calbiochem (Bie & Berntsen, Roedovre, Denmark). CEP 1347 and CEP 11004 were kindly provided by Cephalon (West Chester, PA, USA).

Animals

Ventral mesencephalon (VM) tissue was derived from E14 Sprague Dawley rat embryos (Taconic M & B, Ry, Denmark). In all *in vivo* experiments, adult female Sprague Dawley rats (Taconic M & B) weighing 220–250 g at the beginning of the experiment were used. Two animals per cage were housed at a 12 h day/night cycle with access to food and water *ad libitum*. All experimental procedures were carried out in accordance with the directives of the Danish National Committee on Animal Research Ethics and the European Communities Council Directive #86/609 for care of laboratory animals.

Experiments in PC12 cells

PC12 cells (ATCC CRL 1721; Greene and Tischler 1976) were maintained in DMEM supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal bovine serum, 1% sodium pyruvate and 1% penicillin/streptomycin. For experiments, cells were plated in collagen G coated, 6 well plates with 100 000 cells/mL (2 mL/well) and left overnight to adhere. The medium was then replaced with medium containing 2% serum (DMEM plus 1% heat inactivated horse serum and 1% heat inactivated fetal bovine serum), MnCl₂ (1 mM) and CEP 1347/CEP 11004 (30–500 nM) were added. For immunocytochemistry, cells were fixed in 3.7% formaldehyde after 6 h and stained for p c jun according to the manufacturer's protocol (c jun activation hit kit). Immunoreactivity was visualized using an Alexa Fluor 488 conjugated secondary antibody included in the kit. Nucleus intensity was determined by image processing software using the 'cytoplasm to nucleus translocation' algorithm on the CCD camera based ArrayScan HCS system (Cellomics). Eight wells were used for one data point, and 200–800 cells were analysed per well.

To quantify the effect of CEP 11004 on Mn²⁺ induced cell death, cells were stained with the fluorescent dye Hoechst 33342 and the number of apoptotic nuclei was scored by use of fluorescence microscopy. Cells with condensed and fragmented nuclei were scored as apoptotic.

For western blotting, cells were lysed after 16 h in 200 µL lysis buffer (10 mM Tris HCl (pH 7.2), 1% NP40, 1 mM AEBSF), and 10 µg protein were loaded onto 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and analysed by phospho specific antibodies against p JNK (1 : 1000) and p c jun (1 : 1000) followed by a HRP conjugated anti mouse antibody. Labelled proteins were detected using ECL Plus reagent.

Preparation of primary rat ventral mesencephalic cell cultures or suspensions

Primary dopaminergic neurones from rat embryonic ventral mesencephalon (VM) were prepared as previously described (Kaminski Schierle *et al.* 1999b). Briefly, VM tissue was dissected from 14 day old rat embryos and kept in ice cold HBSS. The tissue was trypsinized in a solution containing 0.1% (wt/vol) trypsin and 0.05% (wt/vol) DNase dissolved in HBSS. After 20 min incubation at 37°C, the tissue was rinsed four times in a 0.05% DNase HBSS solution. The tissue was mechanically dissociated into a cell suspension. The cells were either used as suspension for transplantation or seeded on poly L lysine (50 mg/L) coated plates for *in vitro* experiments. For culture experiments, neurones were cultured in

DMEM containing 1 mM sodium pyruvate, 15 mM HEPES, 1% penicillin/streptomycin and 10% (v/v) heat inactivated fetal bovine serum. Alternatively, the neurones were cultured in Neurobasal medium supplemented with B27, 0.5 mM L glutamine and 1% penicillin/streptomycin. The cells were seeded in eight well chamber slides at a density of 62 500 150 000 cells/cm². Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cultures were used for up to 7 days *in vitro* (DIV).

In the graft study, some cell suspensions were treated with CEP 1347 (1 µM) during each step of the procedure, including the tissue dissection and dissociation. In all cases, special care was taken to balance the number of embryos and the volumes of solutions to obtain cell suspensions with similar cell concentration.

Cell death models in VM cultures

Three different models were used for *in vitro* experiments. For each model, two to four independent culture experiments were performed. The first model was a 30 48 h culture model that mimics the conditions of the cells during the first phase of a transplantation experiment when there is the highest percentage of cell death (Fawcett *et al.* 1995; Kaminski Schierle *et al.* 1999a). The VM derived cells were plated on chamber slides in medium (DMEM plus 10% serum) containing different concentrations of test compound (CEP 1347). The indicated concentrations of CEP 1347 were also present during all preparation steps, from tissue dissection to plating. When cells had firmly adhered, i.e. 30 48 h after plating, the cultures were fixed with 4% paraformaldehyde and stained using an antibody against TH. Immunoreactivity was detected using an ABCComplex/HRP system and DAB was used as chromogen.

The second model was designed to mimic the conditions of the transplanted cells once they are in the adult brain and slow death occurs due to lack of growth factor support (Engele 1998). Briefly, after 2 DIV, the medium was changed to serum free medium containing various concentration of CEP 1347 (0 1000 nM). After 7 DIV, cultures were fixed with 4% paraformaldehyde and processed for immunostaining of TH. Cultures fixed after 2 DIV served as a control for 100% survival. After 7 DIV, the survival of dopaminergic neurones in the culture was reduced to 15 20%.

The third model was an ageing related slow degeneration model, where test compounds were added to the culture 1 h after plating of the cells. For these experiments, the medium was changed to Neurobasal medium plus supplements and test compound after 1 day in culture. Every third day, two thirds of the medium was changed. After 6 7 DIV, the cultures were fixed and immunostained as described above.

In all models, the experimental endpoint was the number of TH immunopositive neurones/culture. For each experimental group, four to eight culture wells were scored with regard to the number of TH immunopositive neurones. The number of TH immunopositive neurones was counted on blind coded slides using a semi automated stereological cell counting system (Computer Assisted Stereological Test Grid System: Olympus C.A.S.T. Grid system, Albertslund, Denmark) as described previously (Schierle *et al.* 1999).

Activation of the JNK pathway in VM cultures

For western blotting, cells from model 1 were lysed after 24 h in 200 µL lysis buffer (10 mM Tris HCl (pH 7.2), 1% NP40, 1 mM

AEBSF), and 10 µg protein were loaded onto 4 12% SDS polyacrylamide gels. Proteins were transferred to PVDF membranes and analysed by phospho specific antibodies against p JNK (1 : 1000) and p c jun (1 : 1000), followed by a HRP conjugated anti mouse antibody. Labelled proteins were detected using ECL Plus reagent.

Unilateral 6 hydroxydopamine lesion and motor asymmetry test

For all surgical procedures, the rats were anaesthetized with a mixture of Hypnorm (fentanyl/fluanisone, Janssen Pharmaceutical, Belgium) and Dormicum (midazolam, Hoffman La Roche, Switzerland). The anaesthetic mixture was prepared with 25% Hypnorm and 25% Dormicum in sterile distilled water and administered as 2.7 mL/kg BW s.c. For post operational analgesia the rats received Temgesic (0.3 mg/mL buprenorphine, Schering Plough, Farum, Denmark) 1 mL/kg BW s.c. of a 1 : 3 diluted (with sterile saline) solution.

Female Sprague Dawley rats were subjected to unilateral 6 hydroxydopamine (6 OHDA) lesion of the ascending mesostriatal dopamine pathway as described previously (Grasbon Frodl *et al.* 1996). Three weeks post lesion the effect of the 6 OHDA injury was assessed by monitoring amphetamine (D amphetamine sulphate 2.5 mg/kg, i.p.) induced turning behaviour (Ungerstedt and Arbuthnot 1970). Rats exhibiting a net rotation asymmetry (turns contralateral to the lesion subtracted from ipsilateral turns) of at least seven full turns/min were selected for transplantation surgery. Using a 5 mg/kg dose of amphetamine, this turning rate corresponds to a striatal dopamine depletion of ≥ 97% (Schmidt *et al.* 1983). The rotation test was repeated at 2 and 5 weeks after the transplantation surgery.

Transplantation surgery and administration of compound

CEP 1347 was dissolved in a 7.5% solutol/phosphate buffered saline (PBS) vehicle. Vehicle treated rats received a daily injection of the same volume (2 mL/kg) of vehicle. Vehicle/compound administration was started 1 week prior to transplantation and continued until the day before termination 5 weeks post transplantation.

In a single transplantation session, two deposits of 1.5 µL of the cell suspension (in total approximately 250 000 cells equivalent to 50% of one VM) were injected stereotaxically into the right (lesioned) striatum of recipient rats as described previously (Grasbon Frodl *et al.* 1996) and at the following coordinates (from bregma): A 1.0, L 3; V -5 and -4.5 mm, Tooth Bar - 3.3.

Histological assessment

Five weeks after transplantation the rats were deeply anaesthetized with Avertin 10 mL/kg (Bie & Berntsen) and perfused through the heart with saline followed by phosphate buffered 4% (w/v) paraformaldehyde. The brains were post fixed for 4 h in the same fixative, cryoprotected in phosphate buffered 30% sucrose, and then sectioned coronally at 40 µm thickness using a cryostat. Brain sections throughout the graft region were collected in three parallel series. One series was reacted for free floating TH immunohistochemistry with the ABC detection kit and DAB as a chromogen. Sections were mounted on gelatinized slides, air dried, dehydrated in an ascending series of alcohols, cleared with xylene and coverslipped using the mounting medium Pertex (Bie & Berntsen).

The total numbers of TH positive neurones in each graft were stereologically counted on parallel TH immunostained, blind coded

slides using the C.A.S.T. Grid system as described previously (Schierle *et al.* 1999). Using the same system and the same slides, graft volumes and fibre outgrowth were also determined using the Cavalieri principle for volume estimation (Møller *et al.* 1995; Gregersen *et al.* 2000).

Statistics

Cell count data were analysed by one factor ANOVA with group (treatment) as independent variable. Behavioural data that had been collected on several testing sessions pre and post grafting were analysed using repeated measures ANOVA, where time (testing session) and group (treatment) were entered as independent variables. All posthoc comparisons were carried out using the Tukey 'honestly significant difference' method or, when the data distribution was not normal, the Kruskal Wallis one way ANOVA on Ranks and Dunn's method. Statistical significance was set at $p < 0.05$. Data are expressed as group means \pm SD or SEM with indication of the sample size.

Results

Enhancement by CEP-1347 of the survival of cultured dopaminergic neurones prepared for transplantation

First, it was tested whether CEP 1347 had an effect on embryonic dopaminergic cells in conditions simulating the initial phase of the transplantation protocol. After the cell preparation, which is a major cell death inducing stress within the transplantation procedure, the cells were short term cultured in serum containing medium, i.e. optimal culture conditions, and analysed after 40 h. CEP 1347 treatment during preparation and after plating resulted in an increase in TH positive neurones, which was already significant at concentrations as low as 30 nM and reached a maximal effect at 500 nM (Fig. 1a).

We further investigated whether CEP 1347 acts on its supposed target pathway in this cellular model. We found that 24 h after plating, JNK was highly activated as detected by phospho specific antibodies for phospho JNK. In cultures prepared and maintained in the presence of CEP 1347, activation of the JNK pathway was reduced by 83%, as quantified by densitometry and beta actin normalization (Fig. 1b).

Enhancement of survival by CEP-1347 in serum-depleted neurones

In the second set up, cell cultures were stressed by serum withdrawal. This lack of trophic factor resembles the second phase of the transplantation protocol, where the cells have been transplanted but lack the trophic support of their natural environment. It was found that CEP 1347 (30–1000 nM), when given during the period of serum withdrawal, significantly increased the number of TH positive neurones compared to cultures treated with medium alone. Maximal protection was observed at 100 nM (Fig. 2).

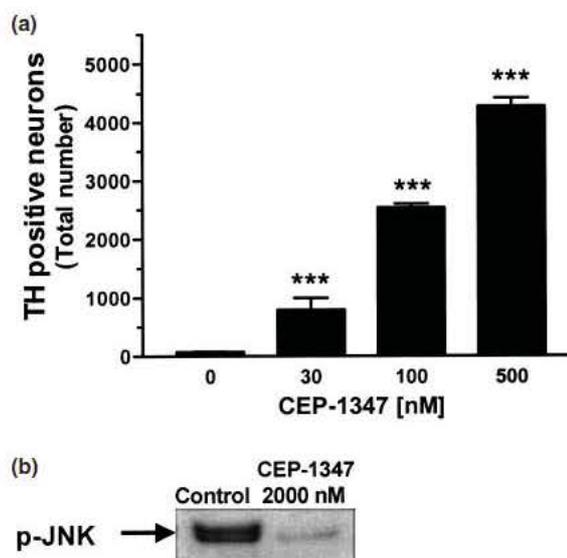


Fig. 1 CEP 1347 blocks activation of the JNK pathway and rescues freshly prepared ventral mesencephalic cells. (a) Protection of dopaminergic neurones by CEP 1347 from cell death occurring during initial preparation steps. Cells were prepared and plated in the presence of the indicated concentrations of CEP 1347 and fixed after 40 h. Surviving TH positive neurones were quantified by stereological counting in 8–11 cultures/data point. Data are means \pm SEM. Asterisks indicate statistical differences from control (** $p < 0.001$, Kruskal Wallis one way ANOVA on Ranks and Dunn's method). (b) Activation of the JNK pathway in dopaminergic cultures is inhibited by CEP 1347. Cells were prepared and plated as mentioned above in the presence of CEP 1347 or vehicle and after 24 h, the cells were lysed and analysed for p JNK by western blotting.

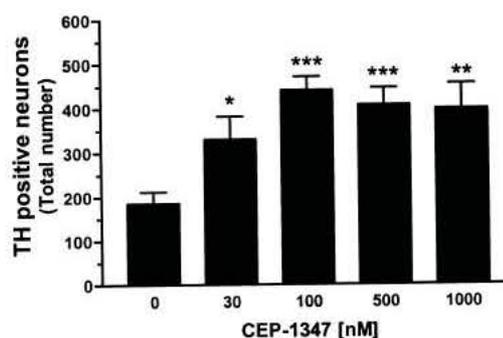


Fig. 2 Rescue of primary dopaminergic neurones by CEP 1347 from stress induced cell death in a serum withdrawal model. Serum was withdrawn from the cultures from day 3–7. During that period CEP 1347 was added to the cultures at the concentrations indicated and surviving neurones were quantified after fixation and TH staining. Asterisks indicate statistical differences from control (* $p = 0.02$; ** $p = 0.003$; *** $p < 0.001$, one way ANOVA and Tukey test, $n = 8$; data represent one experiment expressed as mean \pm SEM).

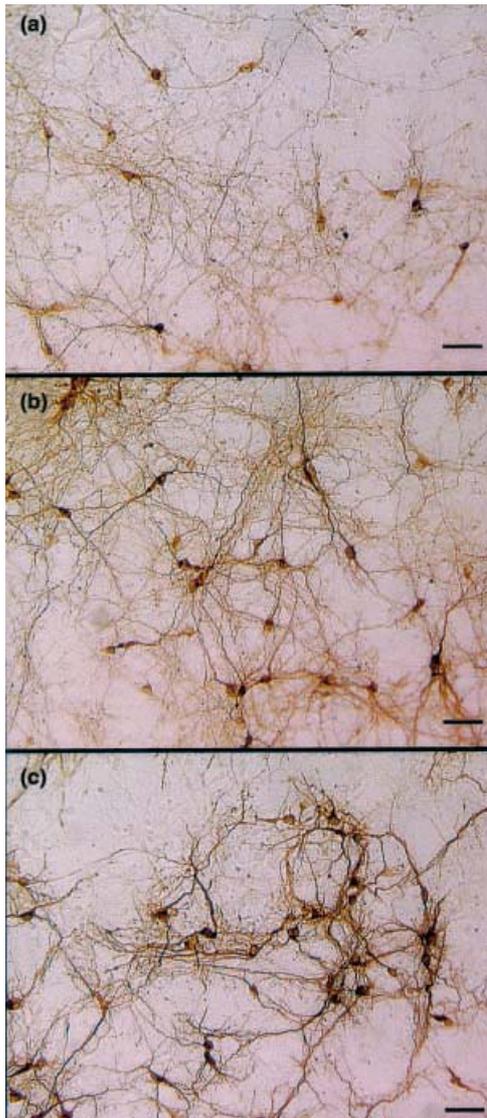


Fig. 3 Morphology of dopaminergic neurones cultured in the presence or absence of CEP 1347. Neurones were cultured in the presence of 0 (a), 100 (b) or 1000 (c) nM CEP 1347 and the cultures were stained for TH after 7 days. The bar indicates 50 µm.

Protection by CEP-1347 from ageing-induced cell death

In the third model, cells were cultured for 7 DIV, and we examined the ability of CEP 1347 to improve overall survival and differentiation of dopaminergic neurones. After 7 DIV, cultures receiving CEP 1347 showed highly increased TH immunoreactivity compared with control cultures (Fig. 3). Most of the increased staining was due to an increased number of TH immunopositive neurones, which was significantly increased by 50–1000 nM of CEP 1347 and more than threefold at 1000 nM (Fig. 4). When looking at individual TH positive neurones, it was evident that drug treatment did not reduce the extent or complexity of the neurite network. On the contrary, CEP 1347 treated neurones

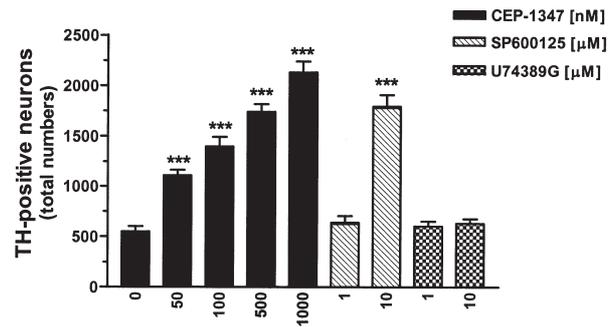


Fig. 4 Enhanced survival of dopaminergic neurones in the presence of CEP 1347 or SP600125, but not Lazaroid U74389G. Neurones were plated and cultured in the presence of the different compounds and the number of surviving neurones was quantified after 7 days by counting of TH positive cells. Asterisks indicate statistical differences from control (***) $p < 0.001$, one way ANOVA and Tukey test, $n = 8$; data represent one experiment expressed as mean \pm SEM).

often appeared to display a more healthy and complex neuronal morphology (Fig. 3). In this model, we also tested the effect of direct inhibition of JNK in order to compare it with the effect of the upstream MLK inhibitor CEP 1347 (Ip and Davis 1998). For this purpose, two commercially available inhibitors of JNK were tested. SP600125 at 10 µM, but not 1 µM, significantly increased the number of TH positive neurones comparable to the increase observed with CEP 1347 (Fig. 4). By contrast, the cell permeable peptide, JNK inhibitor I (1 or 10 µM), did not increase TH immunoreactivity (data not shown). Finally, in this same model, the effects of CEP 1347 were compared with those of a lazardoid (Nakao *et al.* 1994; Hall 1997), the only drug class used for enhancement of cell survival in human transplantation (Brundin *et al.* 2000b). It was examined whether the lazardoid U74389G would protect TH positive neurones from death induced by ageing in culture. Lazardoid (0.3–10 µM) treated cultures did not show any improvement in survival from control cultures (Fig. 4), clearly indicating higher efficacy of CEP 1347 to rescue cells in this model.

Generally protective effects of MLK inhibition in transplantation-linked dopaminergic cell stress

CEP 11004, a second selective MLK inhibitor suitable for culture studies, has recently been described (Murakata *et al.* 2002). We took advantage of the availability of this tool to obtain independent proof for the role of the MLK pathway in dopaminergic cell death. The main cell culture experiments were repeated in the presence of CEP 11004 instead of CEP 1347 and similar protection was observed (Fig. 5).

As a further approach to address the role of the MLK pathway for dopaminergic cell death, we used a model of Mn²⁺ induced stress in PC12 cells. The oxidative stress caused by Mn²⁺ models the stress supposedly occurring during transplantation, and the PC12 cell line offers the

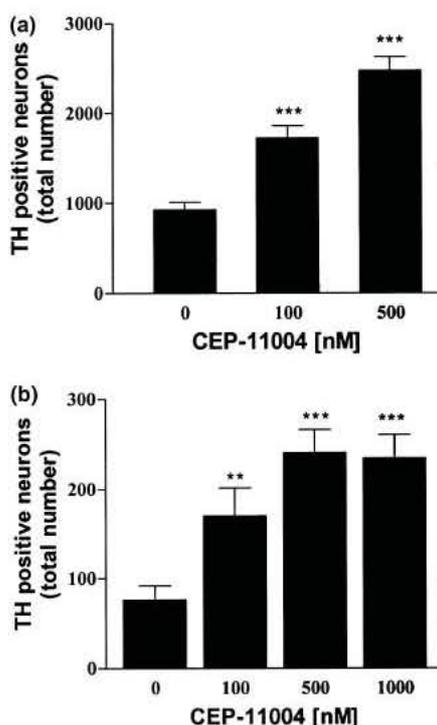


Fig. 5 Enhanced survival of dopaminergic neurones in the presence of CEP 11004. (a) Neurones were plated and cultured in the presence of the indicated concentrations of CEP 11004 and the number of surviving neurones was quantified after 7 days by counting of TH positive cells. Asterisks indicate statistical differences from control (** $p < 0.001$, one way ANOVA and Tukey test, $n = 4$; data represent one experiment expressed as mean \pm SEM). (b) Rescue of primary dopaminergic neurones by CEP 11004 from serum withdrawal induced cell death. Serum was withdrawn from the cultures from day 3–7. During that period CEP 11004 was added to the cultures at the concentrations indicated and surviving neurones were quantified after fixation and TH staining. Asterisks indicate statistical differences from control (** $p = 0.01$; *** $p < 0.001$, one way ANOVA and Tukey test, $n = 4$; data represent one experiment expressed as mean \pm SEM).

advantaged of a homogenous cell preparation. Treatment with $MnCl_2$ activated JNK and caused activation of c-jun, and this was blocked by both CEP 1347 and CEP 11004 (Fig. 6). These results obtained by western blotting were confirmed by single cell immunofluorescence analysis, which clearly showed that CEP 11004 completely blocked the Mn^{2+} triggered phospho c-jun translocation from cytoplasm to the nucleus. Block of c-jun activation was paralleled by a significant decrease in apoptotic cell death.

Effects of treatment with CEP-1347 on the survival and function of dopaminergic neurones in mesencephalic grafts

We tested whether treatment of a VM cell suspension with CEP 1347 would enhance the survival of dopaminergic neurones after intrastriatal grafting, and whether treatment of

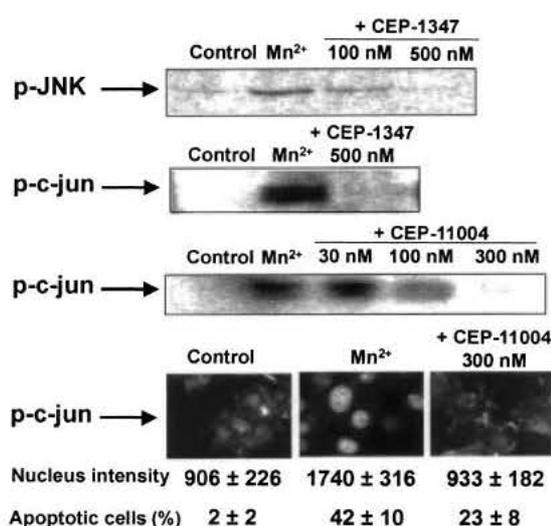


Fig. 6 The role of the MLK pathway in Mn^{2+} induced stress in PC12 cells. The rat dopaminergic PC12 cells were incubated with CEP 1347/CEP 11004 and challenged with 1 mM $MnCl_2$. After 16 h, cells were lysed and analysed for p JNK and p c-jun levels by western blotting (top 3 panels). After 6 h, cells were fixed and stained for p c-jun by immunocytochemistry (lower panel). The nuclear staining intensity of p c-jun was determined by densitometric analysis of the fluorescent staining in the nucleus. Data below are quantifications from $n = 8$ wells with 200–800 cells analysed per well and given in arbitrary units \pm SD. CEP 11004 significantly decreased the nuclear staining intensity ($p < 0.001$, one way ANOVA and Tukey test). In parallel cultures, the percentage of apoptotic nuclei was counted after 24 h by use of fluorescence microscopy. CEP 11004 significantly decreased the percentage of apoptotic nuclei ($p < 0.001$, one way ANOVA and Tukey test, $n = 4$). The data are presented as percentage of total number of cells (mean \pm SD) from four independent experiments.

the recipient host with the compound would enhance such an effect.

Recipient rats were divided into four groups of six animals each: (i) control group, receiving cells treated with vehicle and daily injection of vehicle; (ii) 'CEP 1347 cells' group, receiving cells treated with 1 μ M CEP 1347 and daily injection of vehicle; (iii) 'CEP 1347 host' group receiving cells treated with vehicle and daily injection of CEP 1347 (0.3 mg/kg s.c.); (iv) 'CEP 1347 cells plus host' group receiving cells treated with CEP 1347 (1 μ M) and daily injection of CEP 1347 (0.3 mg/kg s.c.).

In all animals that received intrastriatal injection of VM cell suspensions, we detected at 5 weeks post grafting the presence of large grafts in the striatum containing TH immunopositive neurones (not illustrated). Although in all animals the main body of the grafts was in the striatum, in several of them the grafts were not completely contained within this structure, and also extended into the overlying cerebral cortex. In all animals, a dense network of TH immunopositive fibres extended from the graft into the surrounding striatum and cerebral cortex (not illustrated).

Stereological counts of TH positive neurones showed that there was a higher number of surviving dopaminergic neurones in all animals of the CEP 1347 treated groups compared with those of the vehicle group. This increase in survival was most distinct (> 250% more neurones) for the group with combined treatment of the cell suspension and daily host administration of CEP 1347 ('CEP 1347 cells plus host'). This observed significant increase in survival was due to a clear increase in the mean number of surviving neurones (Fig. 7a), as well as to a substantial decrease in the variability in number of surviving neurones in different animals (3276 ± 1043 cells in vehicle group vs. 8297 ± 404 in the 'CEP 1347 cells plus host' group). When graft volumes were examined, we found a clear graft increasing effect of

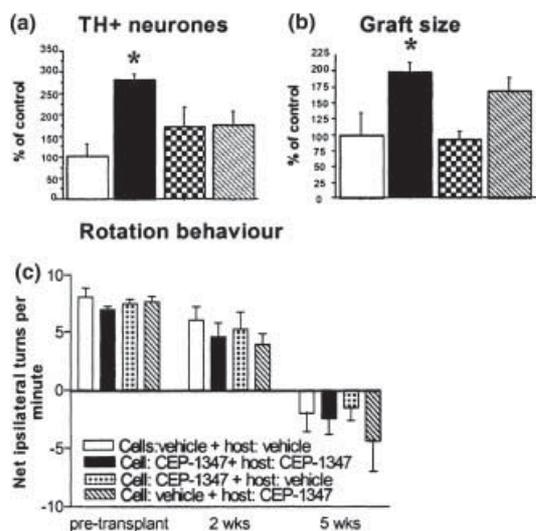


Fig. 7 Effects of CEP 1347 on morphological parameters and functional outcome of grafting of embryonic dopaminergic neurones in the denervated striatum of hemiparkinsonian rats. Cells used for transplantation were incubated with 0 or 1 μM CEP 1347 and animals were treated daily with vehicle or 0.3 mg/kg CEP 1347 as indicated. The transplants were examined 5 weeks after surgery. (a) The total number of surviving TH immunopositive neurones was counted by stereological methods. The number of TH positive neurones in controls was 3276 ± 1043 and the results are expressed as percentage of vehicle (control) treated grafts. Asterisks indicate statistical differences from control ($*p < 0.05$, ANOVA with posthoc Tukey test). (b) Graft size. Results are expressed as percentage of vehicle (control) treated grafts (the volume of control grafts was 0.756 ± 0.261 mm³). Asterisks indicate statistical differences from control ($*p < 0.05$, ANOVA with posthoc Tukey test). (c) Rotation behaviour. Before transplantation, and 2 and 5 weeks after, the amphetamine induced turning behaviour of the hemiparkinsonian rats was tested and 'net ipsilateral turns per minute' were scored. Negative values at the axis mean 'contralateral turns', i.e. overcompensation by the graft for cells previously lost by the 6 OHDA treatment. Statistical analysis of group time interaction indicated that extent and rate of recovery between 2 and 5 weeks is not significantly different between the grafted groups (two factors ANOVA, effect of group $p > 0.05$).

CEP 1347 treatment of the recipient rats (Fig. 7b), reaching a significance level in the 'CEP 1347 cell plus host' group in comparison with the vehicle group and the 'CEP 1347 cells' group. We also examined the extent of outgrowth of TH positive fibres from the grafts and found that this parameter was also improved by CEP 1347 treatment of recipient rats, although not to significance level, most likely because of some variability of this parameter (data not shown). We further tested whether CEP 1347 treatment would result in enhanced functional recovery as detected in the amphetamine induced rotation test. At 2 weeks post transplantation, all grafted groups showed a decrease in their rotation score compared with the pre operation values, with a reduction to around 75% of the pre transplantation score in the vehicle group and up to around 50% in the group where both cell suspension and host were treated with compound. At 5 weeks post transplantation, all groups exhibited reversal of motor asymmetry. This is an indication that in all rats there were very large functioning transplants (confirming the data of the counts of TH positive neurones and measurement of graft size reported in Fig. 7a and b). A bigger negative score (= contralateral rotations) indicates a larger transplant. Also, at this survival time the group with suspension plus recipient treated with CEP 1347 showed a trend towards better performance than vehicle treated rats (Fig. 7c). Statistical analysis of group time interaction, however, indicated that extent and rate of recovery between 2 and 5 weeks were not significantly different between the grafted groups (two factors ANOVA, effect of group $p > 0.05$).

Discussion

In this study we have shown that CEP 1347, an MLK inhibitor, improves the survival of primary embryonic dopaminergic neurones both in culture in four different stress models and *in vivo* after transplantation into the denervated striatum of adult hemiparkinsonian rats. In this last condition, to obtain the best survival of the transplanted dopaminergic neurones, it is necessary to combine treatment of the cell suspension and of the host. We also showed that in PC12 cells and in VM cultures exposed to stress similar to that during graft preparation, there is activation of the JNK pathway, which is inhibited by both CEP 1347 and CEP 11004.

CEP 1347 and CEP 11004 were neuroprotective against a wide range of insults. The extent of beneficial effects of the MLK inhibitors was in the same range as that reported for established neuroprotectants for embryonic dopaminergic neurones, members of the lazard family [our data and Karlsson *et al.* (2002)] and caspase inhibitors (Schierle *et al.* 1999; Hansson *et al.* 2000). This suggests that MLK mediated JNK activation is a critical upstream event in stress induced death of primary embryonic dopaminergic neurones.

It is a point of concern that inhibition of JNK may also have negative effects on neurones by preventing physiological and differentiation signals. However, in our *in vitro* experiments, the cultures treated with CEP 1347 looked morphologically indistinguishable from control cultures or, frequently, even 'healthier', i.e. displaying a larger and more elaborate neurite network. Similar results have been obtained in trophic factor deprived sympathetic neurones (Harris *et al.* 2002b). A possible reason for this is that CEP 1347 does not block JNK directly, but block only one of several JNK activation pathways. MLK inhibitors specifically block suprabasal stress induced JNK activity while leaving the physiological basal cellular JNK activity intact (Mielke and Herdegen 2002). It has recently been shown that one of the mechanisms by which protein kinase B/AKT promotes cell survival is via phosphorylation of MLK3, leading to its inhibition (Barthwal *et al.* 2003). Thus, inhibition of MLK may be a very 'physiological' way of blocking cell death, leading to only minimal disruption of other vital cell pathways.

In our study, we showed that MLK inhibition improves the survival of primary dopaminergic neurones not only in culture but also after grafting in the adult brain. We obtained the best protection with combined treatment of the suspension and of the host. With this combined treatment the percentage of surviving neurones in comparison with controls was similar to that obtained by combined treatment of lazaroïd plus caspase inhibitors (Hansson *et al.* 2000), or of caspase inhibitors and complement inhibition (Cicchetti *et al.* 2002). This suggests that MLK inhibition can block several of the mechanisms involved in the death of transplanted neurones both during the very first phase of transplantation and later when the neurones are long term exposed to the adult CNS environment. We also found that the neuroprotective action of CEP 1347 is not only restricted to the dopaminergic neurones but also to other cells present in the graft, since we detected a significant increase in total graft size with compound treatment. Again, we found that treatment of the host was critical for this effect, since the group with combined treatment had significantly larger grafts than both the control and the 'CEP 1347 cells' groups. The fact that treatment of the host is critical for best protection may indicate the need for continuous exposure of the grafted neurones to the inhibitor to block their death. It may also imply that the compound has some effects on the brain milieu surrounding the graft, increasing its permissiveness to cell survival, and this will require further investigation. There was also a trend towards a positive effect of CEP 1347 on the fibre outgrowth and on functional outcome of the grafting procedure. However, our study was focused on the clearly measurable survival effect and was not designed and powered to examine functional effects, which depend on many experimental parameters specific for the model chosen. The position of the grafts in our study

was not completely contained in the striatum and thus, not in the best location for optimal fibre outgrowth. Moreover, for functional studies, the number of transplanted cells needs to be carefully titrated down so that untreated grafts remain too small to yield full reversal of rotational asymmetry. As shown by our rotation experiments, even control grafted animals compensated after 5 weeks, which indicates potent, possibly saturating graft function without drug treatment. Comparison of the number of grafted neurones with the number of survivors after 5 weeks shows that even after CEP 1347 treatment, the majority of grafted dopaminergic neurones still die (52.6%, determined as described in Nakao *et al.* 1994). This may be due to the fact that MLK inhibition does not block all types of JNK mediated cell death (Salehi *et al.* 2002), or that other death pathways are involved. On the other hand, as has been shown for cultured cerebellar granule cells (Harris *et al.* 2002a), inhibition of death pathways in the absence of activation of survival pathways may not be sufficient for long term survival of the grafted neurones. Further studies with combined treatment of grafts (and ideally also their hosts) with drugs able to act on all these mechanisms are necessary to answer these questions.

Finally, it has also been shown that transplantation prevents the differentiation of dopaminergic precursors present in the grafts into a dopaminergic phenotype (Sinclair *et al.* 1999). Treatments aimed at promoting such differentiation, combined with a treatment to promote survival and/or block death of the grafted neurones, may thus be necessary to obtain the best outcome of the grafting procedure.

CEP 1347 has been found to be neuroprotective in animal models of PD (Saporito *et al.* 1999) and is a compound currently in clinical Phase 2 trials for PD. Thus, it is attractive to speculate that when used in clinical practice to promote survival of dopaminergic neurones grafted in parkinsonian patients it may, as a bonus, protect the dopaminergic neurones of the grafted host, leading to long term improvement of the disease.

Acknowledgements

We gratefully acknowledge the skilled technical support of Anne Klit Thomsen and Lone Lind Hansen.

References

- Barker R. A., Dunnett S. B., Faissner A. and Fawcett J. W. (1996) The time course of loss of dopaminergic neurons and the gliotic reaction surrounding grafts of embryonic mesencephalon to the striatum. *Exp. Neurol.* **141**, 79–93.
- Barthwal M. K., Sathynarayana P., Kundu C. N., Rana B., Pradeep A., Sharma C., Woodgett J. and Rana A. (2003) Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. *J. Biol. Chem.* **278**, 3897–3902.

- Bozyczko-Coyne D., O-Kane T. M., Wu Z. L., Dobrzanski P., Murthy S., Vaught J. L. and Scott R. W. (2001) CEP-1347/KT-7515, an inhibitor of SAPK/JNK pathway activation, promotes survival and blocks multiple events associated with Abeta-induced cortical neuron apoptosis. *J. Neurochem.* **77**, 849–863.
- Branton R. L. and Clarke D. J. (1999) Apoptosis in primary cultures of E14 rat ventral mesencephala: time course of dopaminergic cell death and implications for neural transplantation. *Exp. Neurol.* **160**, 88–98.
- Brundin P., Isacson O. and Bjorklund A. (1985) Monitoring of cell viability in suspensions of embryonic CNS tissue and its use as a criterion for intracerebral graft survival. *Brain Res.* **331**, 251–259.
- Brundin P., Karlsson J., Emgard M., Schierle G. S., Hansson O., Petersen A. and Castilho R. F. (2000a) Improving the survival of grafted dopaminergic neurons: a review over current approaches. *Cell Transplant.* **9**, 179–195.
- Brundin P., Pogarell O., Hagell P. *et al.* (2000b) Bilateral caudate and putamen grafts of embryonic mesencephalic tissue treated with lazarooids in Parkinson's disease. *Brain* **123**, 1380–1390.
- Choi W. S., Yoon S. Y., Oh T. H., Choi E. J., O'Malley K. L. and Oh Y. J. (1999) Two distinct mechanisms are involved in 6-hydroxydopamine- and MPP⁺-induced dopaminergic neuronal cell death: role of caspases, ROS, and JNK. *J. Neurosci. Res.* **57**, 86–94.
- Chun H. S., Gibson G. E., DeGiorgio L. A., Zhang H., Kidd V. J. and Son J. H. (2001) Dopaminergic cell death induced by MPP⁺, oxidant and specific neurotoxicants shares the common molecular mechanism. *J. Neurochem.* **76**, 1010–1021.
- Cicchetti F., Costantini L., Belizaire R., Burton W., Isacson O. and Fodor W. (2002) Combined inhibition of apoptosis and complement improves neural graft survival of embryonic rat and porcine mesencephalon in the rat brain. *Exp. Neurol.* **177**, 376–384.
- Emgard M., Karlsson J., Hansson O. and Brundin P. (1999) Patterns of cell death and dopaminergic neuron survival in intrastriatal nigral grafts. *Exp. Neurol.* **160**, 279–288.
- Engel J. (1998) Spatial and temporal growth factor influences on developing midbrain dopaminergic neurons. *J. Neurosci.* **53**, 405–414.
- Fawcett J. W., Barker R. A. and Dunnett S. B. (1995) Dopaminergic neuronal survival and the effects of bFGF in explant, three dimensional and monolayer cultures of embryonic rat ventral mesencephalon. *Exp. Brain Res.* **106**, 275–282.
- Freed C. R., Greene P. E., Breeze R. E. *et al.* (2001) Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N. Engl. J. Med.* **344**, 710–719.
- Gearan T., Castillo O. A. and Schwarzschild M. A. (2001) The parkinsonian neurotoxin, MPP⁺ induces phosphorylated c-Jun in dopaminergic neurons of mesencephalic cultures. *Parkinsonism Relat. Disord.* **8**, 19–22.
- Grasbon-Frodl E. M., Nakao N. and Brundin P. (1996) The lazarooid U-83836E improves the survival of rat embryonic mesencephalic tissue stored at 4 degrees C and subsequently used for cultures or intracerebral transplantation. *Brain Res. Bull.* **39**, 341–347.
- Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl Acad. Sci. USA* **73**, 2424–2428.
- Gregersen R., Lambertsen K. and Finsen B. (2000) Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. *J. Cereb. Blood Flow Metab.* **20**, 53–65.
- Hagell P. and Brundin P. (2001) Cell survival and clinical outcome following intrastriatal transplantation in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **60**, 741–752.
- Hall E. D. (1997) Lipid antioxidant neuroprotectants for acute and chronic neurodegenerative disorders, in *Neuroprotection in CNS Diseases* (Bär, P. R., Beal, M. F., eds), pp. 161–181. Marcel Dekker, New York.
- Hansson O., Castilho R. F., Kaminski Schierle G. S., Karlsson J., Nicotera P., Leist M. and Brundin P. (2000) Additive effects of caspase inhibitor and lazarooid on the survival of transplanted rat and human embryonic dopamine neurons. *Exp. Neurol.* **164**, 102–111.
- Harper S. J. and LoGrasso P. (2001) Signalling for survival and death in neurons: the role of stress-activated kinases, JNK and p38. *Cell Signal.* **13**, 299–310.
- Harris C., Maroney A. C. and Johnson E. M. Jr (2002a) Identification of JNK-dependent and -independent components of cerebellar granule neuron apoptosis. *J. Neurochem.* **83**, 992–1001.
- Harris C. A., Deshmukh M., Tsui-Pierchala B., Maroney A. C. and Johnson E. M. (2002b) Inhibition of the c-Jun N-terminal kinase signaling pathway by the mixed lineage kinase inhibitor CEP-1347 (KT7515) preserves metabolism and growth of trophic factor-deprived neurons. *J. Neurosci.* **22**, 103–113.
- Ip Y. T. and Davis R. J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK) from inflammation to development. *Curr. Opin. Cell Biol.* **10**, 205–219.
- Kaminski Schierle G. S., Hansson O. and Brundin P. (1999a) Flunarizine improves the survival of grafted dopaminergic neurons. *Neurosci.* **94**, 17–20.
- Kaminski Schierle G. S., Hansson O., Ferrando-May E., Nicotera P., Brundin P. and Leist M. (1999b) Neuronal death in nigral grafts in the absence of poly (ADP-ribose) polymerase activation. *Neuroreport* **10**, 3347–3351.
- Karlsson J., Emgard M., Gido G., Wieloch T. and Brundin P. (2000) Increased survival of embryonic nigral neurons when grafted to hypothermic rats. *Neuroreport* **11**, 1665–1668.
- Karlsson J., Emgard M. and Brundin P. (2002) Comparison between survival of lazarooid-treated embryonic nigral neurons in cell suspensions, cultures and transplants. *Brain Res.* **955**, 268–280.
- Kordower J. H., Freeman T. B., Chen E. Y., Mufson E. J., Sanberg P. R., Hauser R. A., Snow B. and Olanow C. W. (1998) Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Mov. Disord.* **13**, 383–393.
- Lindvall O. and Hagell P. (2000) Clinical observations after neural transplantation in Parkinson's disease. *Prog. Brain Res.* **127**, 299–320.
- Maroney A. C., Glicksman M. A., Basma A. N. *et al.* (1998) Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. *J. Neurosci.* **18**, 104–111.
- Maroney A. C., Finn J. P., Connors T. J. *et al.* (2001) Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. *J. Biol. Chem.* **276**, 25302–25308.
- Mielke K. and Herdegen T. (2002) Fatal shift of signal transduction is an integral part of neuronal differentiation: JNKs realize TNF α -mediated apoptosis in Neuronlike, but not naive PC12 cells. *Mol. Cell. Neurosci.* **20**, 211–224.
- Møller A., Christophersen P., Drejer J., Axelsson O., Peters D., Jensen L. H. and Nielsen E. Ø. (1995) Pharmacological profile and anti-ischemic properties of the Ca²⁺-channel blocker NS-638. *Neurol. Res.* **17**, 353–360.
- Murakata C., Kaneko M., Gessner G. *et al.* (2002) Mixed lineage kinase activity of indolocarbazole analogues. *Bioorg. Med. Chem. Lett.* **12**, 147–150.
- Nakao N., Frodl E. M., Duan W. M., Widner H. and Brundin P. (1994) Lazarooids improve the survival of grafted rat embryonic dopamine neurons. *Proc. Natl Acad. Sci. USA* **91**, 12408–12412.
- Piccini P., Brooks D. J., Bjorklund A. *et al.* (1999) Dopamine release from nigral transplants visualized in vivo in a Parkinson's patient. *Nat. Neurosci.* **2**, 1137–1140.

- Salehi A. H., Xanthoudakis S. and Barker P. A. (2002) NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway. *J. Biol. Chem.* **277**, 48043–48050.
- Saporito M. S., Brown E. M., Miller M. S. and Carswell S. (1999) CEP-1347/KT-7515, an inhibitor of c-jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons *in vivo*. *J. Pharmacol. Exp. Ther.* **288**, 421–427.
- Saporito M. S., Thomas B. A. and Scott R. W. (2000) MPTP activates c-Jun NH (2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons *in vivo*. *J. Neurochem.* **75**, 1200–1208.
- Schierle G. S., Hansson O., Leist M., Nicotera P., Widner H. and Brundin P. (1999) Caspase inhibition reduces apoptosis and increases survival of nigral transplants. *Nat. Med.* **5**, 97–100.
- Schmidt R. H., Bjorklund A., Stenevi U., Dunnett S. B. and Gage F. H. (1983) Intracerebral grafting of neuronal cell suspensions. III. Activity of intrastriatal nigral suspension implants as assessed by measurements of dopamine synthesis and metabolism. *Acta Physiol. Scand. Suppl.* **522**, 19–28.
- Sinclair S. R., Fawcett J. W. and Dunnett S. B. (1999) Dopamine cells in nigral grafts differentiate prior to implantation. *Eur. J. Neurosci.* **11**, 4341–4348.
- Sortwell C. E., Pitzer M. R. and Collier T. J. (2000) Time course of apoptotic cell death within mesencephalic cell suspension grafts: implications for improving grafted dopamine neuron survival. *Exp. Neurol.* **165**, 268–277.
- Tabbal S., Fahn S. and Frucht S. (1998) Fetal tissue transplantation in Parkinson's disease. *Curr. Opin. Neurol.* **11**, 341–349.
- Ungerstedt U. and Arbuthnott G. W. (1970) Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res.* **24**, 485–493.
- Xu Z., Maroney A. C., Dobrzanski P., Kukekov N. V. and Greene L. A. (2001) The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol. Cell Biol.* **21**, 4713–4724.
- Zawada W. M., Zastrow D. J., Clarkson E. D., Adams F. S., Bell K. P. and Freed C. R. (1998) Growth factors improve immediate survival of embryonic dopamine neurons after transplantation into rats. *Brain Res.* **786**, 96–103.