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Both apoptosis and necrosis occur early after intracerebral grafting of ventral mesencephalic tissue: a role for protease activation

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Abstract
Neural transplantation is an experimental treatment for Parkinson’s disease. Widespread clinical application of the grafting technique is hampered by a relatively poor survival (around 10%) of implanted embryonic dopamine neurones. Earlier animal studies have indicated that a large proportion of the grafted cells die during graft tissue preparation and within the first few days after intracerebral implantation. The present study was designed to reveal the prevalence of cell death in rat intrastratal grafts at 90 min, 1, 3, 6 and 42 days after implantation. We examined apoptotic cell death using semithin and paraffin sections stained with methylene blue and an antibody against activated caspase 3, respectively. We identified abundant apoptotic cell death up to 3 days after transplantation. In addition, we studied calpain activation using an antibody specific for calpain-cleaved fodrin. We report a peak in calpain activity 90 min after grafting. Surprisingly, we did not observe any significant difference in the number of dopaminergic neurones over time. The present results imply that grafted cells may be victims of either an early necrotic or a later apoptotic cell death and that there is substantial cell death as early as 90 min after implantation.

Keywords: apoptosis, calpain, necrosis, neural transplantation, Parkinson’s disease, substantia nigra.


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Abbreviations used: FBDP, fodrin breakdown product; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labelling.
Expressed as a percentage of the number of dopamine neurones in the harvested donor tissue, the highest survival rates achieved so far have only been in the range of 40–50% (Hansson et al. 2000; Clarke and Branton 2002; Sortwell 2003). Several studies have focused on limiting cell death by counteracting apoptosis (for review see Brundin et al. 2000 and Castilho et al. 2000; Duan et al. 2000; Espejo et al. 2000; Hansson et al. 2000; Helt et al. 2001; Zawada et al. 2001; Cicchetti et al. 2002), while other modes of cell death have been addressed less systematically (Cicchetti et al. 2002; Zietlow et al. 2002).

Caspases and calpains are two groups of cysteine proteases known to participate in mechanisms of cell death. Caspases are considered major executors of apoptosis, whereas pathological activation of calpains is considered more important in necrosis. Apoptosis is a fundamentally important process whereby non-functional cells can be eliminated with a minimum of inflammation. Caspases are at the heart of the apoptotic pathways and have been named in order of publication (Nicholson and Thornberry 1997; Wolf et al. 1999), at present caspase 1 to 14. Treatment with high concentrations of the caspase inhibitor acetyl-Tyr-Val-Ala-Asp-chloro methylketone has been shown to increase the survival of grafted dopaminergic neurones (Scherle et al. 1999b; Hansson et al. 2000). However, at the concentrations employed, acetyl-Tyr-Val-Ala-Asp-chloro methylketone is likely to have also inhibited other proteases in the graft tissue (Hansson et al. 2000; Gray et al. 2001). Calpains are a group of Ca2+-activated neutral cysteine proteases present in all mammalian cells (Croall and DeMartino 1991; Saido et al. 1994; Vanderklish and Bahr 2000). Calpain activity is strictly regulated by cytosolic calcium and interaction with, e.g. the endogenous inhibitor protein calpastatin and membrane phospholipids. Under pathological conditions, calpains have mainly been implicated in excitotoxic neuronal injury (Huang and Wang 1994; Vanderklish and Bahr 2000). The aims of the present study were to investigate cell death in intrastriatal grafts shortly after transplantation surgery and to shed further light on the mechanisms underlying the cell death process. Specifically, we monitored the occurrence of degenerating neurones as soon as 90 min after graft injection and followed the implants for up to 6 weeks. Markers of caspase and calpain activation, as well as methylene blue-stained semi-thin sections, were followed throughout the time course to monitor the demise of cells in the tissue cell graft and in the host brain following transplantation.

Materials and methods

Animals and chemicals

Intrastriatal transplantations of embryonic ventral mesencephalic tissue were performed as described previously (Engård et al. 1999). All animals were purchased from B & K Universal (Solletuna, Sweden). The experimental procedure was ethically approved and animals were handled according to the animal protection act of the Swedish Government. All chemicals were purchased from Sigma (Stockholm, Sweden) if not otherwise stated.

Preparation of donor tissue

Embryonic dopaminergic neurones were obtained from the ventral mesencephalon of 14-day-old (crown-to-rump length 12 mm) rat embryos, where the day after mating was considered as day zero. The pregnant rats were killed with sodium pentobarbitone (200 mg/kg body weight i.p.) and the uterus removed by Caesarean section. The embryos were dissected under sterile conditions in Ca2+-free Hank’s balanced salt solution (Gibco, Life Technologies, Täby, Sweden) and the tissue pieces were incubated with 0.1% trypsin (Worthington, Lakewood, NJ, USA)/0.05% Dnase in Hank’s balanced salt solution for 20 min at 37°C. After incubation, the ventral mesencephalon tissue pieces were washed four times in 0.05% Dnase in Hank’s balanced salt solution and mechanically dissociated using a fire-polished Pasteur pipette. The resulting cell suspension, with a volume equal to one ventral mesencephalon in 6 μL, contained a mixture of single cells and small tissue aggregates.

Intrastriatal transplantations

Adult female Sprague–Dawley rats (weighing 220 g at the start of the experiment) were anaesthetized with a mixture of Hypnorm (fentanyl citrate and fluanisone, 0.213 mg and 6.75 mg/kg bodyweight, respectively; Janssen Pharmaceutical, Breese, Belgium) and Dormicum (midazolam, 3.375 mg/kg bodyweight; Hoffman-La Roche, Basel, Switzerland). The rats were placed in a Kopf stereotactic apparatus and tissue injections were made with the following coordinates (in mm, with reference to the bregma and dura): rostral, +1.0; lateral, +3.0; ventral, −5.0 and 4.5; with tooth-bar, 0. Injections were performed at a rate of 1 μL/min using a 10-μL Hamilton syringe, fitted with a cannula with a 0.26-mm inner diameter and 0.46 mm outer diameter. Each rat received a total of 3 μL cell suspension, equivalent to one half ventral mesencephalon. The cannula was left in place for 2 min between deposits and an additional 4 min before withdrawal after the last implantation. To evaluate damage in the host striatum caused by the needle injection, in a separate set of animals, sham-transplantations were made with injections of 3 μL of Hank’s balanced salt solution. Temgesic (0.3 mg/mL Buprenorphine solution; 0.17 mg/kg body weight s.c.; Reckitt and Colman, Hull, UK) was administered immediately after surgery for analgesia.

Tissue preparation

The survival times for both tissue-transplanted and sham-grafted rats were 90 min, 1, 3, 6 or 42 days. Rats were deeply anaesthetized with sodium pentobarbitone (200 mg/kg body weight, i.p.) and
perfused with 4% paraformaldehyde as previously described (Engård et al. 1999). The animals (tissue or sham grafted) that were further prepared for semi-thin sections were perfused with 2% glutaraldehyde. The brains were post-fixed for 1 day in the same fixative.

Semi-thin sections
For the semi-thin sections, a vertical tube of the transplanted hemisphere was dissected out and further fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer. The tissue blocks were rinsed, dehydrated in graded concentrations of ethanol and rinsed in propylenglycol. The tissue blocks were further incubated overnight in a 1:1 mixture of proplyenoxide and Agar 100 resin solution (consisting of Agar 100 epoxy resin, dodecylsuccinic anhydride, methyl Nadic anhydride and benzylidemethylamine; Agar Link Nordiska AB, Upplands Vasby, Sweden), followed by an incubation for 6 h in pure Agar 100 epoxy resin. Finally, the tissue blocks were allowed to polymerize for 3 days at 60°C. The plastic-embedded tissue blocks were sectioned sagittally into semi-thin, i.e. 1–2 μm, sections. Azur (1%) and methylene blue (1% in 1% borax) were used as Nissl stain and the cell morphology of the grafted neurones was studied.

Paraffin sections
After dehydration with graded ethanol concentrations and xylene, the brains were paraffin embedded and cut into 5-μm coronal sections using a microtome. The sections were divided into 10 series. Each series constituted immediate adjacent sections, with 12–15 sections per brain and series. Before immunohistochemical staining, the sections were incubated for 30 min at 65°C and deparaffinized with xylene and graded series of ethanol. Antigen retrieval was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min.

Immunohistochemistry

Tyrosine hydroxylase immunohistochemistry
Endogenous peroxidase activity was quenched with 3% H₂O₂ followed by incubation with with 2% normal rabbit serum (ICN Biochemicals, Aurora, OH, USA) in 1% Triton X-100 for 1 h. A monoclonal mouse anti-tyrosine hydroxylase (TH; Chemicon, Hampshire, UK) was added onto the sections (1:2000) and incubated in a humidified box overnight at 4°C, followed by a Cy3-conjugated rabbit anti-mouse secondary antibody (1:50; Jackson Immuno-Research, West Grove, PA, USA) for 1 h at room temperature. Sections were mounted with polyvinyl-alcohol in diaza-bicyclo-octane (ICN Biochemicals).

Fodrin breakdown product immunohistochemistry
The sections were pre-incubated for 30 min in 4% normal goat serum followed by incubation in a humified box at 4°C overnight with a polyclonal antibody against the 150 kDa calpain-specific fodrin breakdown product (FDBP, 1:50; Bahr et al. 1995). A biotinylated goat anti-rabbit IgG (1:136; Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody. Sections were incubated for 1 h at room temperature, followed by 10 min with 3% H₂O₂ in phosphate-buffered saline. After rinsing, the sections were incubated with avidin–biotin complex (Vectastain Elite; Vector Laboratories) for 1 h at room temperature. Chromogen development was performed with nickel-enhanced diamino benzidine. Sections were mounted with Histofix (Histolab, Göteborg, Sweden).

Active caspase 3 immunohistochemistry
The sections were pre-treated with 10 μg/mL proteinase K (Roche Diagnostics, Mannheim, Germany) for 10 min at room temperature before incubation with 4% normal goat serum for 1 h. The polyclonal primary antibody against active caspase 3 (1:50; PharMingen, Becton Dickinson AB, Stockholm, Sweden) was incubated in a humidified box at 4°C overnight, followed by a 1 h incubation with a biotinylated secondary goat anti-rabbit antibody (1:500; Vector Laboratories). Quenching of endogenous peroxidase activity and development with avidin–biotin complex and diamino benzidine was performed as described above.

Double staining
Two different double stainings were carried out on one representative brain per time point (90 min, 1, 3 and 6 days), combining TH and FDBP or TH and caspase 3. Staining procedures were initiated with staining for TH (as described above) with a red fluorescent goat anti-mouse secondary antibody (1:100; Alexa Fluor 594; Molecular Probes, Eugene, OR, USA). Staining for FDBP and active caspase 3 was performed as described above with a green fluorescent goat anti-rabbit secondary antibody (1:400; Alexa Fluor 488). Sections were mounted with fluorescent mounting medium (DAKO Corporation, Carpinteria, CA, USA).

Microscopic evaluation
The TH-, FDBP- and caspase 3-positive cells within the grafts were counted manually and the total number of cells was calculated according to Abercrombie (1946). In the sections stained for TH, positively stained cells without condensed chromatin or granular appearance were counted. The graft–host border in the FDBP- and caspase 3-stained sections was distinguished by comparing with the immediately adjacent TH-stained sections. The morphology of the grafted cells and the presence of erythrocytes were assessed in the semi-thin sections. Apoptotic cells were considered to be cells with highly condensed chromatin. Brain sections were evaluated using a BX60 microscope (Olympus, MicroMacro AB, Göteborg, Sweden) and photographs taken with a DP50 cooled digital camera (Olympus). Double-stained sections were evaluated in a fluorescence microscope and the number of cells double stained for TH and FDBP or TH and caspase 3 was counted in all the sections in the series.

Statistics
All statistical analyses were performed using statview (version 5.0). One factor analysis of variance (ANOVA) was used to compare several different groups and was followed by Scheffe’s post-hoc test to evaluate individual group differences.

Results

Semi-thin sections through transplants
Cells with apoptotic morphology, i.e. cells with highly condensed chromatin, were observed within the grafts at the
four earliest time points (Figs 1a–e). Evaluation of the number of apoptotic cells per section revealed a significant peak at 1 day after implantation compared with the other time points ($F(4,9) = 12.2$, $p = 0.0011$) (Fig. 2a). We also observed numerous neutrophils and erythrocytes that were characterized according to their morphologies in the oedematous core of the transplants, which largely consisted of a fluid-filled cavity, up to 6 days after transplantation. In brain sections from animals that received sham transplants we observed few scattered apoptotic cells in the striatum adjacent to the implant, primarily at the 90 min and 1 day time points (data not shown). The vehicle sham injections caused tissue damage and oedema in the host cortex and striatum along the needle tract, similar to tissue implantation.

**Tyrosine hydroxylase immunohistochemistry**

Every tenth section was stained for TH. We observed TH-positive neurones at all time points examined, i.e.
90 min, 1, 3, 6 and 42 days after implantation (Figs 1f–j and u). There was no significant difference over time in the number of TH-positive neurones \((F(4,14) = 0.42, p > 0.05)\) (Fig. 2b). Staining for TH appeared to be weaker at early time points after grafting and gradually increased in intensity at later times (Figs 1f–j). At 90 min and 1 day after surgery, the cell bodies were smaller in size with few, if any, neurites (Fig. 1u).

**Fodrin breakdown product immunohistochemistry**

We observed numerous FBDP-immunopositive cells in grafts early after transplantation (Figs 1k–o and v). Statistical analysis revealed a significant difference over time regarding the number of cells positive for FBDP in the grafts \((F(4,14) = 12.9, p = 0.0001)\) (Fig. 2c). Post-hoc analysis with Scheffé’s test showed no difference between the 90 min and 1 day time points \((p > 0.05)\) but there was a difference between these two early time points compared with both 6 and 42 days after surgery \((p < 0.05)\). The 3-day group differed from the 1-day group \((p < 0.05)\) but not from the 90 min time point \((p > 0.05)\). The magnitude of FBDP staining in the recipient brains surrounding the graft site was similar to that observed in sham-injected animals (data not shown). Thus, in addition to the numerous cells labelled in the grafts, there was also a rim of FBDP-positive cells in the striatum adjacent to the graft and in the cortex along the cannula tract.

**Active caspase 3 immunohistochemistry**

Cells positive for caspase 3 were observed from 90 min to 6 days after transplantation (Figs 1p–t and x). Scheffé’s post-hoc test revealed no difference between 1 day and either 90 min, 3 or 6 days post implantation \((p > 0.05)\) but there was a significant difference between 1 and 42 days \((F(4,14) = 5.0, p = 0.01)\) (Fig. 2d). Similar to semi-thin sections and sections stained for FBDP, there were some degenerating cells (positive for caspase 3) along the needle tract up to 3 days after grafting in the sham-operated animals (data not shown).

**Double stainings**

Cells positive for both TH and FBDP or TH and caspase 3 were observed up to 6 days after grafting (Fig. 3). The number of TH-positive cells that were also positive for FBDP or caspase 3 changed over time, similar to the changes in the total number of FBDP- and caspase 3-positive cells, i.e. largest at 90 min and 1 day, respectively. The number of TH-positive cells that were also positive for FBDP decreased from 64.2% at 90 min to 53% at 1 day, 10% at 3 days and 15.8% at 6 days. The percentage of TH-positive cells double-positive for caspase 3 was initially 11.9%, increased to

![Fig. 3](image)
23.6% 1 day after implantation, 28.4% after 3 days and decreased after 6 days to 6.1%.

Discussion

In the present study we evaluated cell death in embryonic nigral transplants at different time points between 90 min and 6 weeks after implantation into the striatum of adult rats. Initially, we examined grafts that had been embedded in epoxy plastic, cut into semi-thin sections and stained with azur and methylene blue. This technique provided two advantages. First, the internal structure of the brain sections was well preserved. In contrast, previous studies, e.g. using free-floating frozen sections (Duan et al. 1995; Emgård et al. 1999) or paraffin-embedded sections (Sortwell et al. 2000), describe that during the first few days after tissue implantation large portions of the graft tissue seem to be missing and fall out from the sections. Second, in semi-thin sections labelled with routine stains it is possible to discern, e.g. detailed nuclear morphology in individual cells. Thereby, apoptotic profiles can be observed and information obtained regarding ongoing cell death in the grafts. At 90 min after surgery, the earliest time point examined, the central parts of the grafts typically were devoid of nucleated cells. Instead, we found fluid-filled cavities that contained scattered erythrocytes, reminiscent of the description of implanted strands of embryonic nigral tissue provided by Zawada et al. (1998). Thus, previous reports, describing that tissue sections fall apart in the striatum if brains are sectioned soon after transplantation surgery (Duan et al. 1995; Emgård et al. 1999; Sortwell et al. 2000), may have partly misinterpreted their findings. What was earlier described as an artifact of tissue preparation probably represents an oedematous area in the host striatum, secondary to the injection of a significant volume of vehicle together with the grafted cells. In agreement with Zawada et al. (1998), we found that during the first day after surgery the grafted cells adhered to the walls of the fluid-filled cavity. Several earlier studies have pointed out that in mature nigral grafts the dopaminergic neurones tend to be located around the periphery of the transplant tissue (see, e.g. Duan et al. 1995; Emgård et al. 1999). This has been suggested to be due to selective cell death of dopaminergic neurones in the centre of the grafts due to poor trophic support or migration of the dopaminergic neurones from central portions to the periphery (Emgård et al. 1999). The current study does not exclude such events but suggests that the core of the graft site may initially be completely devoid of neural cells.

We have previously described the temporal evolution of cell death in nigral transplants between 6 and 42 days after implantation surgery using Fluoro-Jade as a marker for dying/dead cells (Emgård et al. 1999). We found that Fluoro-Jade-labelled cells were most frequent at the earliest, 6-day time point. The semi-thin sections in the present study provided us with evidence for widespread cell death in the transplants much sooner after transplantation. We observed numerous cells with condensed nuclei at 1 and 3 days after surgery and some degenerating cells were also apparent at 6 days. These observations agree with several previous reports that have described cells positive for terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labelling (TUNEL) (Zawada et al. 1998; Schierle et al. 1999a; Duan et al. 2000; Sortwell et al. 2000; Helt et al. 2001) between 1 and 4 days after surgery. The observations of cell death soon after graft implantation stimulated us to further characterize the type of cell death occurring in the nigral grafts by using specific cell death markers.

Caspase 3 is one of the major executor caspases in the cell death cascade. Caspase substrates are typically structural proteins or proteins involved in cellular homeostasis and repair (for review see Chan and Mattson 1999). The immature brain retains its apoptotic machinery to a larger extent than the adult brain, at least as judged by the presence of caspase 3 (Ni et al. 1998; Blomgren et al. 2001). Immature grafted neurones may, therefore, be more prone to apoptotic death while terminally differentiated neurones are more likely to die by necrosis (Hu et al. 2000). In the semi-thin sections, we observed a peak in apoptotic cell death 1 day after transplantation. In addition, apoptotic cell death was detected using immunohistochemistry for active caspase 3, showing marked staining at 1 and 3 days after grafting. We obtained similar results (data not shown) using a hairpin probe oligonucleotide, which has been shown to correlate with caspase 3 activation (Zhu et al. 2000), as an alternative marker for apoptotic neurones. Several different triggers may have initiated this apoptotic cell death. For cells dying soon after implantation, it is likely that the graft tissue preparation itself has triggered a delayed apoptosis (Emgård et al. 2002). This may either be due to severe mechanical stress (Fawcett et al. 1995) and metabolic perturbation (Castilho et al. 2000) during tissue dissociation or to loss of specific cell contacts, known to induce special forms of programmed cell death (anoikis) in other cell systems (Sakai et al. 2000; Ling et al. 2001). At later time points, the triggering of apoptosis may have been generated by the host brain environment (Sinclair et al. 1999; Karlsson et al. 2000; Zietlow et al. 2002). The presence of toxic cytokines and an unfavourable ionic milieu in the traumatized striatum, as well as an absence of appropriate neurotrophic factors, are all conceivable triggers of apoptosis.

Our results obtained using the antibody against active caspase 3 are essentially in agreement with earlier findings demonstrating apoptotic cells in grafts during the first 1–2 weeks after transplantation and a decline in their numbers over time (Zawada et al. 1998; Sortwell et al. 2000, 2001). Earlier studies on apoptosis in nigral transplants have used TUNEL as the primary marker for dying cells (Mahalik et al. 1994; Zawada et al. 1998; Schierle et al. 1999a; Duan et al. 2000). However, TUNEL is a technique that only labels dying cells, and the use of an antibody against active caspase 3 allows the evaluation of a larger number of dying cells.
1999a; Duan et al. 2000; Sortwell et al. 2000, 2001; Helt et al. 2001). For example, Sortwell et al. (2000) found significantly more TUNEL-positive cells at 4 days than at 7 and 28 days. Similarly, Schierle et al. (1999a) and Duan et al. (2000) reported relatively low numbers of TUNEL-positive cells in nigral grafts at 2 and 4 days after surgery, respectively. There is an extensive loss of dopaminergic neurons in nigral grafts (Brundin et al. 1998; Sortwell et al. 2000) and yet relatively low numbers of cells are positive for either TUNEL or caspase 3. This can be interpreted in many different ways. First, a large proportion of the dopaminergic neuroblasts may have died during the graft tissue preparation or before 90 min after implantation. Indeed, earlier work suggests that there is significant cell death, including apoptosis, already during the tissue preparation steps (for review see Brundin et al. 2000; Emgård et al. 2002). Second, the time it takes for a cell to undergo apoptosis, from the onset of nuclear DNA fragmentation until it is cleared by a phagocyte, has been estimated to be as short as 1–3 h (Gavrieli et al. 1992). Therefore, only small numbers of cells would be expected to be caspase 3- or TUNEL-positive at any given time, even if apoptosis is a major contributor to cell death. Third, it is possible that apoptosis is not the major route of cell death followed by degenerating cells in mesencephalic transplants (Hurelbrink et al. 2001) and that necrosis contributes significantly to the demise of grafted cells. Indeed, even though several earlier studies have reported increased survival by inhibition of apoptosis, the protection is not complete. For this reason we were interested in studying markers of cell death not primarily associated with apoptosis.

Calpains have been implicated in excitotoxic neuronal injury and necrosis (Simon and Noszék 1988; Seubert et al. 1989; Saito et al. 1994; Blomgren et al. 1995) and pharmacological inhibitors of calpains exert a cerebroprotective effect (Lee et al. 1991; Rami and Kriegstein 1993; Bartus et al. 1994). Fodrin is a well-known calpain substrate and detection of specific FBDPs has been widely used to detect calpain activity (Saiman et al. 1984; Nixon 1986; Roberts-Lewis et al. 1994; Saito et al. 1994; Bahr et al. 1995; Blomgren et al. 1999, 2001; Emgård et al. 2002). Calpain activity is high in the developing brain (Blomgren and Karlsson 1989; Blomgren et al. 1995) but FBDP is not detectable in normal adult brain tissue (e.g. Seubert et al. 1989).

The number of FBDP-positive cells in the graft was already high 90 min after transplantation and remained at this level for at least 24 h. After 3 days the number was dramatically reduced and FBDP-positive cells were virtually non-existent after 42 days. This suggests that calpain activation is primarily involved in the death of grafted cells during the first 24 h after transplantation. The coexistence of FBDP- and caspase 3-positive cells at the early time points may either indicate that necrotic and apoptotic processes take place in parallel or that calpain activation triggers the activation of caspase 3 (Nakagawa and Yuan 2000; Blomgren et al. 2001). A growing body of literature suggests functional connections between calpains and caspases (Wang 2000). Common substrate proteins have been identified, such as fodrin (Nath et al. 1996; Vanags et al. 1996; Jänicke et al. 1998; Wang et al. 1998) and calpastatin (Pörn-Ares et al. 1998; Wang et al. 1998). In a model of neonatal, cerebral hypoxia–ischaemia, two phases of fodrin degradation have been observed, where the early phase entailed mainly production of the calpain-specific 150-kDa FBDP and the later phase also included the caspase 3-specific 120-kDa FBDP (Wang et al. 2001). Hence, the initially simple classification of calpains being involved in cytotoxic and caspases in apoptotic cell death has proven to be far more complex. It can, therefore, be speculated that pharmacological calpain inhibition during tissue preparation and during the first 24 h following grafting would greatly improve graft cell survival.

The number of TH-positive cells in the grafts did not change between 90 min and 42 days after transplantation. We have previously observed that a large proportion of cells in the mesencephalic cell suspensions undergoes degenerative changes before implantation (Emgård et al. 2002) and, therefore, a major portion of the death of TH neurones may be complete by the time they are injected into the host brain. However, there may be a discrepancy between the apparent numbers of immunopositive dopamine neurones and the numbers of neurones carrying the TH gene, from 90 min after graft injection and beyond. Although it is known that, at embryonic day 14, rat nigral neurones express TH at the time of grafting and are still TH immunoreactive after they have been subjected to tissue dissociation (Sauer and Brundin 1991), it is possible that TH expression is down-regulated during the first days after grafting, as previously suggested (Zawada et al. 1998). Indeed, in the present study we observed many weakly stained TH neurones with few neurites up to 6 days after surgery, suggesting that the expression of TH is relatively low during the first days after implantation. Therefore, by using immunohistochemical detection, the number of dopaminergic neurones early after transplantation may be underestimated. Furthermore, some dopaminergic neurones may not have fully expressed their phenotype at the time of grafting and their continued development and expression of TH de novo could compensate for the gradual death of grafted dopaminergic neurones. A similar alternative explanation for the constant numbers of TH-positive neurones in the grafts despite an ongoing cell death is that neurogenesis may take place in parallel and exactly balance the cell loss. Speaking strongly against this possibility are the observations of Sinclair et al. (1999), indicating that genesis of transplanted nigral dopamine is complete in rats by embryonic day 14.

Recent reports suggest that calpains or caspase 3 may not only be activated during cell death but can also be stimulated
during cellular repair and cytoskeletal remodelling. The early calpain activation may not merely indicate necrotic cell death but can be evidence of neurones experiencing stress with resultant cytoskeletal damage (Bahr et al. 2002). It has been suggested by Bahr et al. (2002) that neurones can survive even though they have been subjected to a transient calpain activation. Moreover, activation of caspase 3 was recently proposed to be involved in the neuroprotection seen by preconditioning of brain ischaemia (McLaughlin et al. 2003). There is a possibility that some of the cells in the present report which stained positive for both TH and FBDP or caspase 3 may actually survive. This could, to some degree, explain why we observed a relatively high number of double-positive cells but without a decrease in the total number of dopaminergic cells over time.

In conclusion, the survival and morphology of nigral grafts have not previously been studied in detail in tissue sections as early as 90 min after transplantation. Using plastic or paraffin embedding we were able to successfully monitor cell death in embryonic nigral tissue from 90 min until 6 weeks after grafting. Application of specific cell death markers provided further insight into mechanisms of cell death and our results suggest that not only the apoptotic demise of neurones is important in nigral transplants. Calpain activation also appeared to contribute to the death of grafted cells during the first 24 h following transplantation and, therefore, pharmacological inhibition of calpain activity may prove a fruitful strategy to improve cell survival in nigral grafts.

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