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Reassessment of Caspase Inhibition to Augment Grafted Dopamine Neuron Survival

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One experimental therapy for Parkinson's disease (PD) is the transplantation of embryonic ventral mesencephalic tissue. Unfortunately, up to 95% of grafted neurons die, many via apoptosis. Activated caspases play a key role in execution of the apoptotic pathway; therefore, exposure to caspase inhibitors may provide an effective intervention strategy for protection against apoptotic cell death. In the present study we examined the efficacy of two different caspase inhibitors, caspase-1 inhibitor Ac-YVAD-CMK and caspase-3 inhibitor Ac-DEVD-CMK, to augment mesencephalic tyrosine hydroxylase-immunoreactive (TH-ir) neuron survival in culture and following implantation into the denervated striatum of rats. We report that treatment with Ac-YVAD-CMK provided partial but nonsignificant protection for TH-ir neurons against serum withdrawal in mesencephalic cultures plated at low density, while neither caspase inhibitor promoted TH-ir neuron survival in higher density cultures, simulating graft density. We demonstrate that plating procedures (full well vs. microislands) and cell density directly affect the degree of insult experienced by TH-ir neurons following serum withdrawal. This varying degree of insult directly impacts whether caspase inhibition will augment TH-ir neuron survival. Our grafting experiments demonstrate that Ac-YVAD-CMK does not augment grafted TH-ir neuron survival when added to mesencephalic cell suspensions prior to grafting or to mesencephalic reaggregates for 3 days in vitro prior to transplantation. These experiments provide further evidence of the failure of these caspase inhibitors to augment TH-ir neuron survival. Furthermore, we suggest that cell culture paradigms used to model grafting paradigms must more closely approximate the cell densities of mesencephalic grafts to effectively screen potential augmentative treatments.

Key words: Apoptosis; Parkinson's disease; Transplant; Cell density

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss of dopamine (DA) in the nigrostriatal pathway. One treatment for severe PD is the transplantation of dopaminergic neurons derived from the embryonic ventral mesencephalon (VM). This approach has been shown to provide significant clinical benefits to patients (23,28). Unfortunately, the use of embryonic tissue is plagued by low graft viability (18). Researchers have applied different strategies that attempt to counteract the putative triggers of grafted cell death [for review (35)], such as trophic support supplementation (24,30,40), angiogenic factors (5,29), anti-anoikis factors (21), and antioxidants (17,19), to increase the meager 5–20% survival rate. A large component of cell death following transplantation is due to apoptosis and occurs within the first few days following transplantation (20,34,41). Therefore, interference with apoptosis provides an avenue of intervention that may lead to augmentation of grafted DA neuron survival.

Activated caspases play a role in the apoptotic pathway by specifically cleaving peptides after aspartic acid residues. The caspase family can be divided into three groups based on function. Caspase-1, -4, and -5 are involved in inflammation and cytokine activation, caspase-2, -8, -9, and -10 are involved in the initiation of apoptosis, and caspase-3, -6, and -7 are involved in the execution of apoptosis [for review (37)]. The role of caspase overexpression and inhibition has been explored widely in cell culture studies. Miura and colleagues (25) demonstrated that the overexpression of caspase-1 leads to an increase in apoptosis. Additionally, caspase expression is upregulated in response to various insults, such as neuron exposure to 1-methyl-4-phenylpyridinium (MPP+) in culture (3) or transient cerebral ischemia in vivo (4, 14); in both cases the addition of caspase inhibitors reduced the number of apoptotic cells. Caspase-3 inhibition has been shown to prevent the consequences of methylcyclopentadienyl manganese tricarbonyl (MMT)-induced oxidative stress in DAergic neurons (2). It has been hypothesized that utilization of caspase inhibitors

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to block the activation of caspases and consequent commitment to apoptosis could lead to optimal graft survival of transplanted DA neurons.

There are conflicting reports regarding the ability of caspase inhibitors to 1) inhibit apoptosis in DA neurons and 2) to augment DA neuron survival both in culture and in a grafting paradigm. In one laboratory caspase-1 and -3 inhibitors have been shown to augment tyrosine hydroxylase-immunoreactive (TH-ir) neuron survival after serum withdrawal and in mesencephalic cell suspension grafts (13,32). Conversely, other laboratories have reported that caspase-1 and -3 inhibitors, as well as broad spectrum caspase inhibitors, do not increase TH-ir neuron survival after serum withdrawal or grafting (16,38). These laboratories utilized varying tissue dissociation, plating, cell culture density, and grafting protocols.

In the following experiments we evaluated the effects of Ac-YVAD-CMK, an inhibitor of the proinflammatory caspase-1, and Ac-DEVD-CMK, an inhibitor of the executioner caspase-3, on mesencephalic TH-ir neuron survival under a variety of cell culture paradigms and following implantation into the denervated striatum of rats. We report that treatment with Ac-YVAD-CMK provided partial yet nonsignificant protection for TH-ir neurons from serum withdrawal in mesencephalic cultures plated at low density, while neither caspase inhibitor increased TH-ir neuron survival in higher density cultures. We demonstrate that plating procedures (full well vs. microislands) and cell density directly affect the degree of insult experienced by TH-ir neurons following serum withdrawal. Low-density, full-well-plated cultures experienced over 85% TH-ir neuron loss whereas higher density, microisland cultures experienced significantly less loss (~30%). Our grafting experiments demonstrate that caspase inhibition with Ac-YVAD-CMK did not augment grafted TH-ir neuron survival when added to mesencephalic cell suspensions during tissue dissociation prior to grafting or to mesencephalic reaggregates exposed for 3 days in vitro prior to transplantation. These experiments provide further evidence of the failure of these caspase inhibitors to consistently augment TH-ir neuron survival, and suggest new generations of inhibitors and additional strategies be explored to optimize graft viability.

MATERIALS AND METHODS

Animals

Timed pregnant female Fisher 344 rats provided embryonic day 14 donor mesencephalic tissue for culture and grafting studies. A total of 28 male Fisher 344 rats, 3 months old at time of purchase, were lesioned to serve as transplant recipients. Timed pregnant rats were housed individually and graft recipients were housed in pairs in the TECH 2000 vivarium at Rush-Presbyterian-St. Luke's Medical Center, which is fully AAALAC approved. All procedures involving rats were approved by the Rush-Presbyterian-St. Luke's Medical Center Institutional Animal Care and Use Committee (IACUC).

Dissection and Dissociation

Timed pregnant female rats were deeply anesthetized with pentobarbital (50 mg/kg, IP). Using sterile surgical techniques, an incision was made across the area of the two uterine horns. Both horns were removed and placed in a sterile dish in 0.9% NaCl on ice for subsequent dissection. VM brain regions were dissected using sterile techniques from embryonic day 14 rat fetuses and pooled in a cold, sterile calcium/magnesium-free buffer (CMF) as described previously (7). Cell suspensions of embryonic mesencephalic tissue then were prepared through a series of CMF rinses, incubated in 0.125% trypsin for 10 min at 37°C, rinsed in CMF again, and triturated in 0.004% DNase to disperse the cells into a single cell suspension. Trypan blue was added to a sample of cell suspension and viewed in a hemocytometer to assess cell viability and to determine cell counts. Each mesencephalon yielded approximately 600,000 cells under our dissection parameters. Cell suspensions of greater than 95% viability were used for experiments. Based on previous estimates (9), our dissection parameters yielded approximately 18,500 TH-ir cells per embryo.

Cell Culture Experiments

Mesencephalic cells were prepared in full serum medium [Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum, 6 mg/ml glucose, 2.0 mM glutamine, 100 U/ml penicillin, and 2.5 μg/ml fungizone]. Cells were plated in full wells in poly-D-lysine-coated 48-well plates at a low density of 100,000 cells/cm^2 to reproduce conditions used by Schierle et al. (32). To investigate the effects of plating procedures and cell density on cell culture results, cells were also dry plated as 10-μl microislands (33,39) at a density of 30,000, 15,000, and 7500 cells/5 mm^2 on poly-D-lysine-coated plates. On day 3 in vitro, the medium was changed to hormone-supplemented serum-free medium (HSSF) (equal volumes of DMEM and Ham's F-12 supplemented with 1.0 mM glutamine, 1.0 mg/ml bovine albumin fraction V, 0.1 mg/ml apo-transferrin, 5 μg/ml insulin, 10 nM L-thyroxin, 20 nM progesterone, 30 nM sodium selenite, 10 U/ml penicillin, and 2.5 μg/ml fungizone), containing 0 or 500 μM Ac-YVAD-CMK (caspase-1 inhibitor; Calbiochem, La Jolla, CA) or Ac-DEVD-CMK (caspase-3 inhibitor; Calbiochem) (both dissolved in 0.02% methanol). A group of full-serum plates was fixed on day 3 in vitro; these plates served as the control to which all other groups were compared. Each experiment consisted

of at least four wells per treatment, and each experiment was repeated at least twice.

Cell Culture Immunocytochemistry

On day 7 in vitro, medium was removed from cells, which were then rinsed in Tris buffer (pH 7.3), fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 30 min, and rinsed in Tris again. Nonspecific staining was blocked with 10% goat serum in 0.5% Triton X in Tris for 1 h. Cells were incubated in 1:4000 mouse anti-TH antibody (Chemicon, Temecula, CA) overnight at 4°C. After Tris rinses, cells were incubated in 1:400 biotinylated goat anti-mouse IgG secondary antibody (Chemicon) for 2 h, rinsed again, and followed by 2 h in ABC-peroxidase reagent (Vector, Burlingame, CA) for signal amplification. TH immunoreactivity was visualized using chromagen 3,3′-diaminobenzidine (DAB) and 0.03% hydrogen peroxide. For microisland experiments, TH-ir neurons were counted in each of nine 20× fields at the center of each well, summed, and used for statistical analysis, as described previously (21,33). For cells plated in the entire well, 20× fields spanning the full vertical and horizontal width of the well were manually counted, summed, and used for statistical analysis.

Mesencephalic Reaggregates

Preparation of VM reaggregates followed a protocol modified from Heller and colleagues (15) and recently described by Sortwell et al. (36). Employment of reaggregates allowed for long-term exposure of the VM neurons to the caspase inhibitor prior to transplantation. Briefly, 3,000,000 mesencephalic cells were added to sterile 10-ml Erlenmeyer flasks containing 2.5 ml of 50% striatal oligodendrocyte-type 2 astrocyte (O-2A) conditioned medium with 10% fetal bovine serum (FBS). The striatal O-2A conditioned medium consisted of HSSF medium incubated with striatal O-2A progenitor cells for 24 h. Following incubation, the conditioned medium was harvested, filter sterilized, and stored at −20°C until use. To determine the effects of the addition of caspase inhibitor on TH-ir neuron survival after transplantation, 100 μM Ac-YVAD-CMK was added to the medium of some flasks for the duration of the culture interval. This concentration was chosen because it had been demonstrated previously to enhance grafted TH-ir neuron survival (32). Mesencephalic cell-containing flasks were gassed for 10 min each with a blood gas mixture, stoppered, and placed in a rotary incubator at 90 rpm and 37°C. After 24 h small reaggregates formed and were maintained for a total of 3 days. Medium was changed on day 2, including the addition of more 100 μM Ac-YVAD-CMK, and the flasks were regassed with blood gas mixture.

Lesioning Procedures and Behavioral Assessment

Twenty-eight male Fisher 344 anesthetized rats (30 mg/kg pentobarbital, IP) received two unilateral injections of 6-hydroxydopamine (6-OHDA) at a concentration of 5.0 μg/μl and a rate of 1 μl/min for 2 min, one in the medial forebrain bundle (AP –4.3, ML +1.2, DV –7.5) and the other in the substantia nigra pars compacta (AP –4.8, ML +1.5, DV –7.5) (27). Baseline measures of amphetamine-induced rotations were obtained at 2 weeks following 6-OHDA administration. Animals were given 5.0 mg/kg amphetamine (IP) and placed in bowls where rotational behavior was automatically recorded by a Macintosh rotometer program for 85 min. Animals meeting the criterion of 6 turns/minute or greater (n = 20) were assigned to transplant groups and grafted 2 weeks later. Behavioral assessment was conducted at 4 and 8 weeks after grafting. Lesion extent was verified histologically at the conclusion of the experiment.

Grafting Parameters

Rats to be grafted were divided into four different treatment groups (n = 5 per group). Group 1 was grafted with 300,000 mesencephalic cells immediately after tissue dissociation; group 2 was grafted with 300,000 mesencephalic cells dissociated and grafted in the presence of 100 μM Ac-YVAD-CMK; groups 3 and 4 were grafted with mesencephalic reaggregates representing 300,000 mesencephalic cells that were exposed to either control aggregate medium or to aggregate medium plus 100 μM Ac-YVAD-CMK. Estimates of 300,000 cells within mesencephalic cells in reaggregate form were made by counting the total number of aggregates per flask, and the appropriate number (1/10th of the original 3,000,000 cells/flask) transplanted. All mesencephalic cells utilized within the experiment originated from the same pooled E14 dissection to control for TH-ir neuron number. For groups 1 and 2, mesencephalic cells were counted using a hemocytometer and suspended at a final concentration of 100,000 cells/μl. A total of 300,000 cells was grafted into the striatum (AP +0.7, ML +2.5, DV −6.5) of anesthetized rats at a rate of 1 μl/min for 3 min using a 25-gauge Hamilton syringe. Groups 3 and 4 were grafted after 3 days in vitro. We have previously demonstrated that graft viability is not compromised by holding VM neurons in this reaggregate culture system for 3 days (36). Aggregates were loaded under a dissecting microscope into a 25-gauge spinal needle and implanted to the same striatal coordinates as above.

Graft Morphology

Nine weeks after transplantation, rats were deeply anesthetized (60 mg/kg pentobarbital, IP) and perfused intracardially with 4% PFA in PBS. Brains were removed, postfixed for 24 h in 4% PFA, and then transferred to

30% sucrose in 0.01 M PBS. Brains were frozen on dry ice and sectioned at 35-μm coronal sections using a sliding microtome. Every sixth section through the graft was analyzed for surviving TH-ir neurons. Sections were immunostained with the free-floating method using antisera directed against TH (1:4000, Chemicon) overnight at room temperature. Triton X (0.3%) was added to the Tris buffer during incubations and rinses to permeabilize cell membranes. Following primary incubation, sections were incubated in biotinylated secondary antisera against mouse IgG (Chemicon, 1:400) followed by the Vector ABC detection kit employing horseradish peroxidase. TH-ir neurons were visualized by exposure to 0.5 mg/ml DAB and 0.03% hydrogen peroxide in Tris buffer. Sections were mounted on subbed slides, dehydrated to xylene, and coverslipped in Pro-Texx. Manual counts of TH-ir neurons were made at 20× every sixth section, and the sum of these counts was then adjusted according to the method of Abercrombie (1).

Statistical Analysis

An ANOVA followed by a post hoc Fisher's PLSD was used for statistical comparisons between groups. A repeated-measures ANOVA, with time as the repeated measure, was used to analyze the data obtained from rotational assessments.

RESULTS

Effects of Caspase Inhibitors on TH-ir Neuron Survival In Vitro

There was an effect of serum withdrawal on low-density, full-well 100,000 cells/cm^2 cultures, $F(3, 52) = 26.689$, $p < 0.0001$ (Fig. 1). Addition of 500 μM Ac-YVAD-CMK or Ac-DEVD-CMK to full-well 100,000 cells/cm^2 cultures after serum withdrawal tended to prevent more TH-ir neuron loss than control (HSSF) cultures. This result is consistent with previous reports utilizing the same cell culture parameters (32), although our results did not yield significant differences (Fisher's PLSD $p > 0.05$). In contrast, when Ac-YVAD-CMK or Ac-DEVD-CMK was added to higher density microisland cultures after serum withdrawal there was no effect on the number of TH-ir neurons compared with untreated cultures [$F(11, 123) = 70.671$, $p < 0.0001$ for full serum vs. withdrawal, Fisher's PLSD $p > 0.05$ for caspase inhibitors vs. HSSF] (Fig. 2A–C).

Effect of Cell Density on Impact of Serum Withdrawal

To investigate whether the degree of serum withdrawal insult experienced by mesencephalic DA neurons under varying cell density conditions could impact the caspase inhibitor's effects, we next examined the effect of serum withdrawal on microisland cultures plated at different densities. Serum withdrawal was significantly

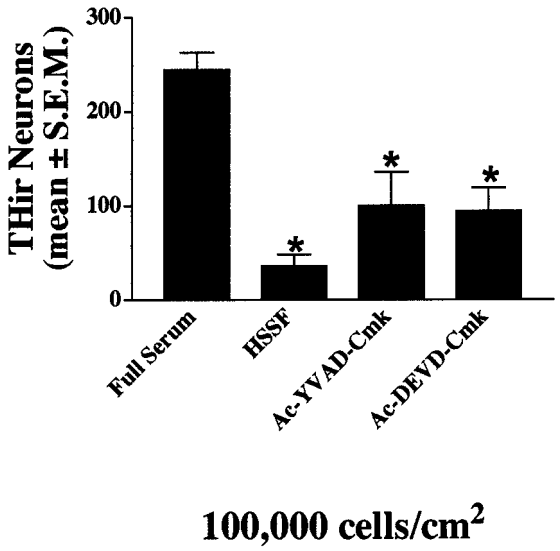

Figure 1. Impact of caspase inhibition on TH-ir neuron survival after serum withdrawal in low-density, full-well cultures. A group of 100,000 cells/cm^2 cultures ($n = 24$) in full-serum medium was fixed at DIV 3 and TH-ir neurons were quantified. On DIV 3 the remaining cultures were switched to a serum-free medium, with 0 ($n = 18$) or 500 μM Ac-YVAD-CMK ($n = 6$) or Ac-DEVD-CMK ($n = 8$), then fixed on DIV 7. Serum withdrawal was significantly detrimental to TH-ir neuron survival. There was a trend towards significance of increased TH-ir neuron survival in caspase inhibitor-treated cultures. *Statistical significance from full-serum cultures ($p < 0.0001$).

detrimental to TH-ir neurons, especially in low-density cultures, $F(7, 108) = 18.135$, $p < 0.0001$ (Fig. 3).

Rotational Asymmetry in Grafted Animals

Amphetamine-induced rotational behavior was used to verify 6-OHDA-induced lesions and assess graft-induced functional recovery (Fig. 4). There were no significant differences in baseline rotational scores between the different transplant groups, $F(11, 48) = 4.667$, $p > 0.05$. At 4 weeks following grafting the fresh control VM group, fresh Ac-YVAD-CMK treated, and the control reaggregate group displayed average ipsilateral rotation rates that were significantly recovered compared with baseline ($p \leq 0.05$). At 8 weeks following grafting all treatment groups except the Ac-YVAD-CMK-treated reaggregate group displayed significant reductions in rotational asymmetry compared with baseline ($p \leq 0.05$). There were no significant differences between Ac-YVAD-CMK-treated suspensions or reaggregates compared with nontreated VM suspensions or reaggregates ($p \leq 0.05$), respectively.

Graft Morphology

Nine weeks posttransplantation all transplant groups exhibited grafts of TH-ir cells within the striatum. Fresh

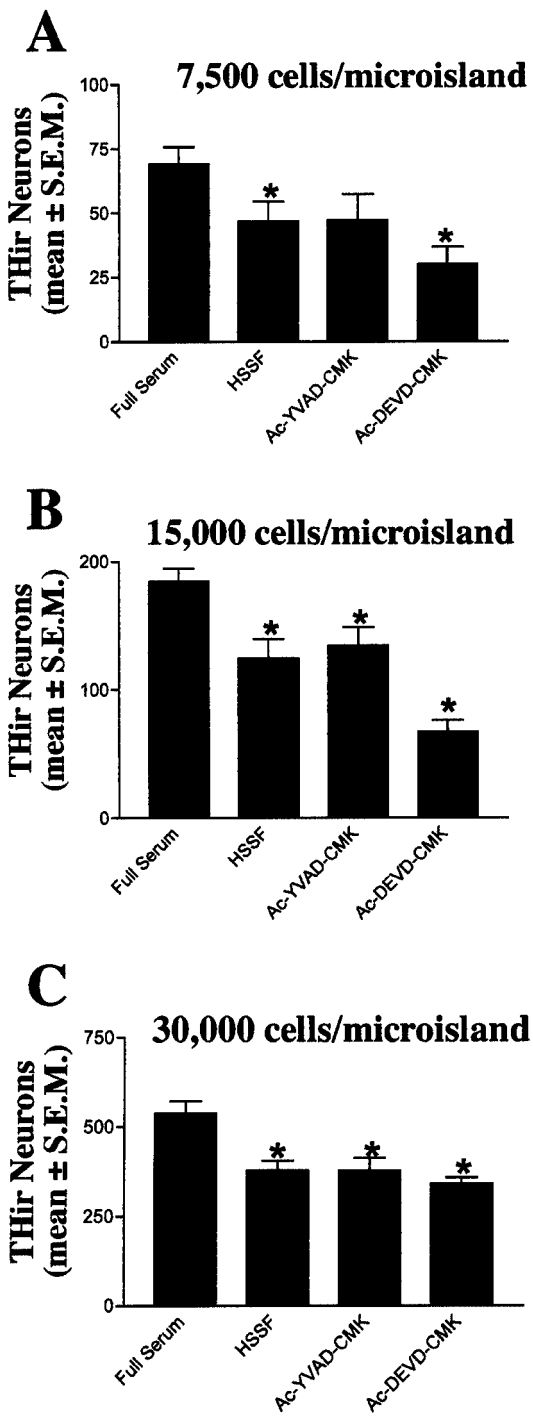

Figure 2. (A–C) Impact of caspase inhibition on TH-ir neuron survival after serum withdrawal in higher density microisland cultures. The addition of Ac-YVAD-CMK to 7500 ($n = 10$), 15,000 ($n = 11$), or 30,000 ($n = 11$) cell microislands did not preserve the number of TH-ir neurons after serum withdrawal. Also, the addition of Ac-DEVD-CMK did not preserve the number of TH-ir neurons after serum withdrawal in 7500 ($n = 11$), 15,000 ($n = 7$), or 30,000 ($n = 11$) cell microislands. *Statistical significance from full-serum TH-ir neuron counts ($p < 0.05$).

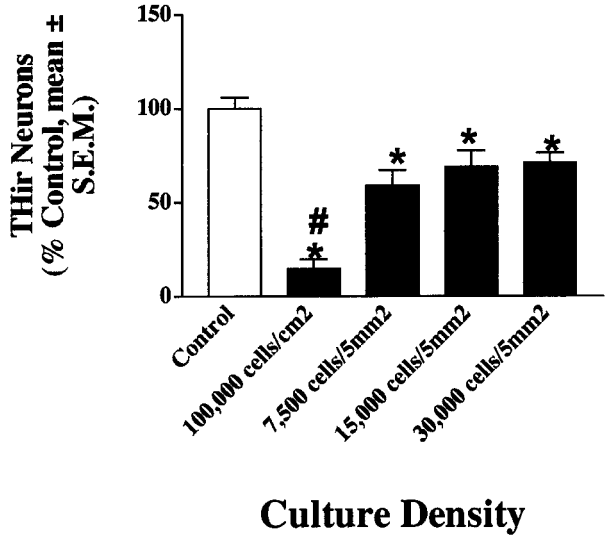

Figure 3. Effect of cell culture density and plating methods on the impact of serum withdrawal. Serum withdrawal was more detrimental to TH-ir neuron survival in low-density cultures. Control is percent of TH-ir neurons in full-serum medium fixed at 3 days in vitro ($n = 54$). TH-ir neuron counts 4 days after serum withdrawal in full-well (100,000; $n = 18$), 7500 ($n = 16$), 15,000 ($n = 15$), and 30,000 ($n = 13$) cells/microisland cultures. Serum withdrawal significantly decreased the number of TH-ir neurons compared with full-serum cultures, especially in low-density cultures. *Statistical significance from full-serum control ($p < 0.05$); #significance from microisland cultures ($p < 0.0001$).

VM suspension grafts displayed an average of 908.4 ± 247.1 surviving TH-ir neurons. This value represents an approximate survival rate of 4.9% of the TH-ir neurons initially transplanted. Ac-YVAD-CM-treated fresh VM suspension grafts displayed an average of 861.33 ± 195.2 TH-ir neurons, representing an approximate survival rate of 4.6%. Reaggregate grafts displayed an average of 822.0 ± 177.10 TH-ir neurons, representing an approximate 4.4% survival rate. Ac-YVAD-CMK-treated reaggregate grafts possessed an average of 810.6 ± 167.8 TH-ir neurons, a 4.4% survival rate. There was no significant difference in the number of grafted TH-ir neurons in any of the four treatment groups ($p > 0.05$) (Figs. 5 and 6).

DISCUSSION

We report that only under specific cell culture conditions in which mesencephalic cells were plated at low densities did the caspase-1 inhibitor Ac-YVAD-CMK or caspase-3 inhibitor Ac-DEVD-CMK display a tendency to increase TH-ir neuron survival in culture after a serum withdrawal insult; however, this trend was not significant. Also, serum withdrawal had a significantly greater impact on low-density cultures, suggesting that

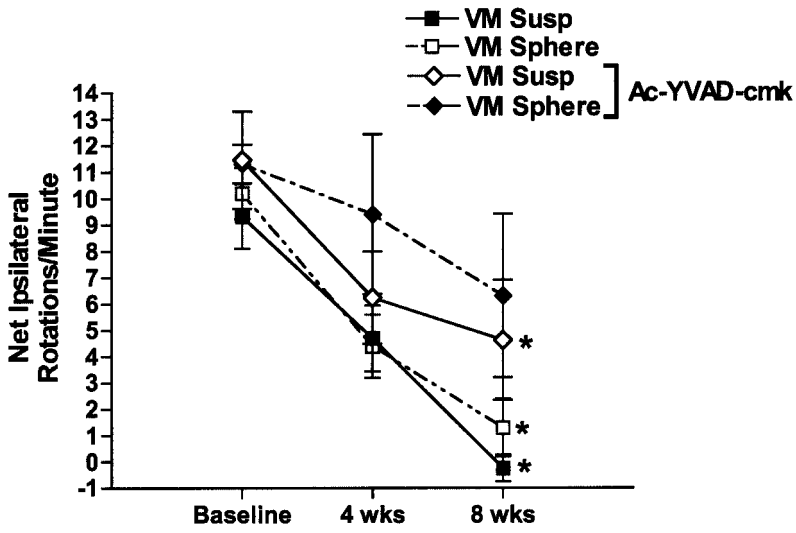

Figure 4. Effect of caspase inhibitor pretreatment on graft-induced recovery from rotational asymmetry. All transplanted animals significantly recovered from baseline at 8 weeks postgrafting, except animals with the AC-YVAD-CMK-treated reaggregates ($n = 5$, per treatment group). There were no differences in rotational asymmetry between any of the treatment groups. *Statistically significant differences from baseline ($p < 0.05$).

density plays a role in cellular stress. The inability of caspase inhibitors to increase the number of TH-ir neurons in high-density cultures is echoed in their failure to augment graft survival and enhance functional recovery when added to either the cell suspension prior to transplantation or when incubated with the cells for 3 days in vitro prior to transplantation. Collectively, our results fail to support the use of these exogenous caspase inhibitors to augment grafted DA neuron survival.

Conflicting reports have been made regarding the ability of exogenous administration of caspase inhibitors to inhibit apoptosis and augment dopamine neuron graft survival. Results likely vary due to the diverse experimental paradigms utilized. Factors that influence caspase inhibitors' efficacy include the degree of insult to which cells are subjected and cell density. Two laboratories have reported significant improvements in TH-ir neuron survival following treatment with caspase inhibitors (6,13,32), while our results and others (16,38) have failed to observe any beneficial effects. The experimental culture paradigm in which Schierle and colleagues (32) observed significant improvements in TH-ir neuron survival with exposure to Ac-YVAD-CMK was in low-density, full-well plating cell culture conditions. Under these exact conditions we also observed a tendency for improvements in TH-ir neuronal profiles with Ac-YVAD-CMK treatment. However, we did not observe improvements in TH-ir neuron number associated with caspase inhibitor treatment under higher density cell culture conditions. This density-related survival phenomenon parallels our findings that examined the role of cell density in determining the impact of serum withdrawal. Low-density (7500 cells/island) microislands experienced a 40.9% loss in TH-ir neurons after serum withdrawal, whereas high-density microislands (30,000 cells/island) only experienced a 28.8% loss. Furthermore, low-density, full-well cultures experienced a robust 85.3% TH-ir neuron loss after serum withdrawal. Our results demonstrate that plating density greatly influences the degree of insult due to serum withdrawal and the ability of the caspase inhibitors to counteract this insult. Sasaki and colleagues (31) demonstrated that in serum-free conditions neuron survival is density dependent due to cell–cell contacts and soluble factors released. They also revealed that apoptosis-inhibiting factors are present in high-density cultures but not low-density cultures (11). These studies suggest that low-density cultures undergo greater baseline cellular stress and therefore are more susceptible to insult and potential rescue than high-density cultures. Furthermore, the method by which cells are plated (dry-plated microislands vs. full well) also impacts cell culture uniformity and density. Microisland dry-plating produces a more uniform density and distribution of cells within the culture well, while cells plated in full wells tend to be most dense at the edges of the plate and relatively sparse in the center. Quantification parameters may also account for variable findings between laboratories (sampling the core of a more uniform island vs. the core of a sparse full-well plate). Our findings indicate that low-density cells cultures are most vulnerable

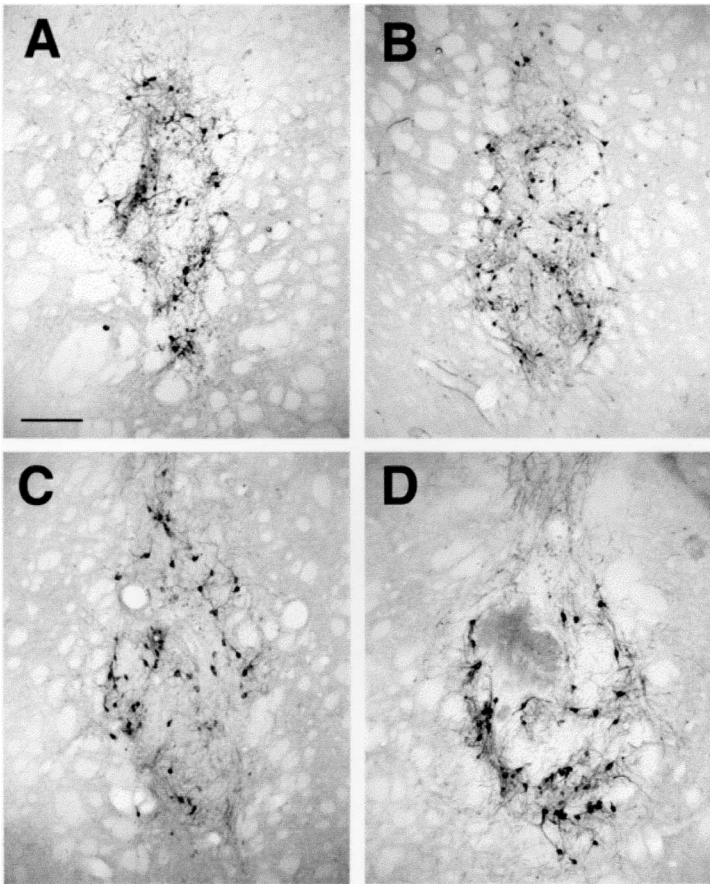

Figure 5. Low-power micrographs of grafted TH-ir neurons with and without caspase inhibitor pretreatment. Grafts of TH-ir neurons are identified with an antibody to tyrosine hydroxylase at 8 weeks posttransplantation into the lesioned striatum. Fresh ventral mesencephalic tissue without (A, $n = 5$) and with AC-YVAD-CMK (B, $n = 5$). Reaggregated mesencephalic tissue without (C, $n = 5$) and with AC-YVAD-CMK (D, $n = 5$) for 3 days in vitro prior to grafting. Scale bar = 100 μM.

to serum withdrawal insult and therefore are most amenable to caspase inhibition augmentation strategies.

Apoptosis is a highly conserved mechanism of cell death with redundant pathways. Therefore, the inhibition of caspase activation does not preclude the execution of other avenues leading to cell death. Even if caspase activation is responsible for the demise of graft viability, clearly other routes of cell death occur. Necrosis has also been shown to play a role in grafted neuron death (8). Prevention of caspase-mediated apoptosis will still leave grafted neurons susceptible to other forms of cell death. Oppenheim and colleagues (26) reported that caspase-3-deficient neurons still die in vivo after genetic deletion, but in a slower time course and via a nonapoptotic pathway. Additionally, it was shown that caspase-3 inhibition delayed, but did not prevent, death of TH-ir neurons after serum withdrawal (38). Moreover, Ac-YVED-CMK and Ac-DEVD-CMK decreased the number of cells with activated caspase-3, but did not augment TH-ir survival in culture (16). In fact, after serum withdrawal, neurons die in an apoptotic-like manner that is caspase independent (12). This corroborates data revealing the rarity of colocalization of TH and activated caspase-3 (16), suggesting that dopamine neurons may die via multiple pathways, some caspase independent. Further evidence that caspase inhibition may not prevent dopamine neuron cells death comes from McLaughlin and colleagues (22), who observed activated caspase-3 without cell death in a model of preconditioning neuroprotection. Their study suggests that mild stressors, which activate caspase-3, also upregulate neuroprotective chaperones such as Hsp70, which can combat insults. Activation of caspases does not unequivocally precede cell death, and inhibition of caspase-dependent apoptotic pathways does not guarantee survival. It appears that the efficacy of caspase inhibitors may be contingent upon

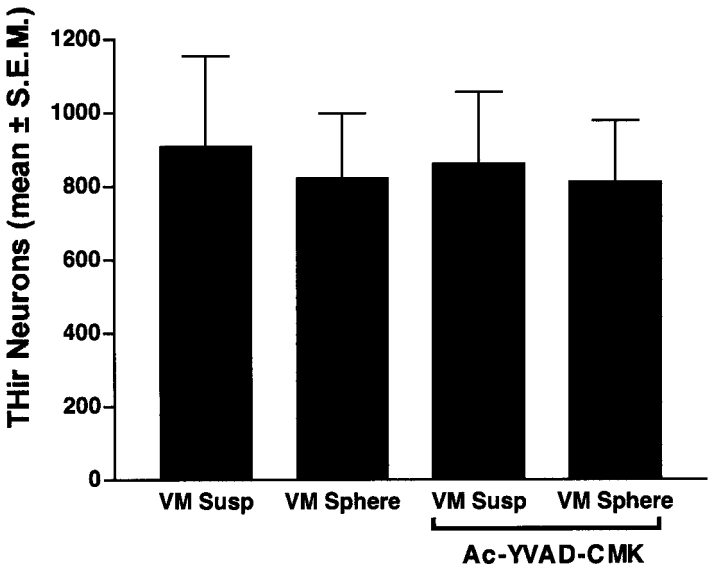

Figure 6. Effect of caspase inhibition on the survival of grafted TH-ir neurons. Quantitation of the number of TH-ir neurons after transplantation into the lesioned striatum. There are no differences in the number of TH-ir neurons in any of the transplanted groups ($p > 0.05$).

experimental paradigm. In summary, DA neuron death induced by various insults can occur in a caspase-independent pathway and caspase activation does not oblige cell death; therefore, the use of caspase inhibitors may be of limited use.

Finally, we report no difference between grafted animal groups in functional recovery, which is consistent with no difference in graft TH-ir counts. As discussed above, it appears that even when caspase-dependent pathways are inhibited, other cell death pathways are used. We have previously shown that there is a significant reduction of apoptotic profiles between days 1, 4, 7, and 28 postgrafting (34). Although we report no difference in grafted TH-ir neuron number between groups, perhaps if caspase inhibitor-treated grafts were examined shortly after transplantation, prior to activation of caspase-independent pathways, it would appear that more TH-ir neurons survived. Additionally, the Schierle et al. (32) study used multiple injection sites with cells at a high density (250,000 cells/1.5 μl, for a total of 500,000 cells), whereas our study used a single injection site at a lower density (300,000 cells/3 μl). It has been shown that there is a switch from apoptotic to necrotic cell death after serum withdrawal in high- and low-density cultures, respectively (10). In the grafting paradigm, it is possible that our lower density grafts are more susceptible to necrosis; therefore, caspase inhibition in not amenable to augment survival. Incongruities in the literature regarding caspase inhibitors' ability to augment dopamine neuron survival are likely due to variations in experimental paradigms.

The results of this study suggest that the efficacy of these caspase inhibitors in preventing apoptosis in DAergic neurons should be reconsidered. Ac-YVAD-CMK and Ac-DEVD-CMK have displayed varying degrees of success both in culture and in vivo in augmenting TH-ir neuron survival after insult or graft implantation. It appears that the efficacy of caspase inhibitors can be contingent upon the experimental paradigm. Unfortunately, there is no standard experimental paradigm. This contributes to the discrepancies in the literature. Clearly, the most beneficial intervention strategies should promote survival under a wide range of conditions. Therefore, the potential of future optimization strategies should be assessed under a variety of cell culture and grafting conditions.

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