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INCREASED CELL SUSPENSION CONCENTRATION AUGMENTS THE SURVIVAL RATE OF GRAFTED TYROSINE HYDROXYLASE IMMUNOREACTIVE NEURONS

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Introduction

Patients with Parkinson's disease (PD) present clinically with bradykinesia, rigidity, and resting tremor (Olanow and Tatton, 1999). While pathology occurs throughout the brain in sporadic PD, these symptoms can mainly be attributed to the degeneration of dopaminergic neurons in the substantia nigra resulting in a loss of striatal dopamine (DA) (Bergman and Deuschl, 2002). Therefore, current therapies center around restoring DA levels in the striatum of affected individuals. Pharmaceutical replacement of DA with levodopa is highly effective as a symptomatic treatment in the early stages of the disease; however, due to the waning efficacy and side effects associated with this therapy, it is not a viable long term treatment option (Jenner, 2004). While animal studies point to the transplantation of embryonic ventral mesencephalic cells as a potential alternative means of restoring DA levels in Parkinson's patients, human trials have yielded mixed results (Brundin, et al., 1987; Freed, et al., 2001; Olanow, et al., 2003; Yurek and Sladek, 1990). Freed et al., 2001, observed a decrease in the total Unified Parkinson's Disease Rating Scale (UPDRS) scores in a subset of younger patients (≤60 years old); however, the transplantation of primary DA neurons in older individuals (>60 years old) did not yield an improvement in the total UPDRS scores (Freed, et al., 2001). A more recent study has shown similar results when comparing preoperative motor scores indicating that it may be the level of disability, rather than age, that influences the level of symptomatic relief derived from the transplantation of primary DA cells (Olanow, et al., 2003). Additionally, the long term side effects of this therapy include debilitating “off period” graft-induced dyskinesias, which are more prevalent in the subset of patients who displayed improvements in Parkinsonian symptoms (Freed, et al., 2001; Olanow, et al., 2003). The limited ability of transplanted DA neurons to survive and properly integrate with the host striatum are likely contributing factors to both the lack of efficacy and side effects associated with the transplantation of embryonic ventral mesencephalic cells.

Numerous factors contribute to the compromised survival rate (5-10%) of DA neurons during the post-transplantation interval including trophic factor withdrawal, hypoxia, and oxidative

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stress (Sortwell, 2003). An additional factor implicated in the low survival rate of transplanted cells is anoikis, i.e. loss of cell-cell and extracellular matrix (EMC) contact (Meredith, 1993; Raff, 1992). Anoikis may play a role in the initiation of apoptosis during the dissociation of the ventral mesencephalon into cell suspension and following transplantation of embryonic tissue into the striatum. These and other insults may also decrease the functional capacity of the surviving tyrosine hydroxylase immunorecative (THir) neurons. Treatment of transplanted DA neurons with neurotrophic factors such as BDNF, NT-3, GDNF, aFGF, and bFGF has been shown to increase the number and functional capacity of grafted THir neurons (Giacobini, et al., 1993; Granholm, et al., 1997; Rosenblad, et al., 1996; Timmer, et al., 2004; Yurek, et al., 1996). However, this approach is only able to increase survival rates to approximately 30-35%, still leaving poor survival as a major obstacle preventing transplantation of primary mesencephalic tissue from becoming a viable therapy for PD (Sortwell, 2003).

While neurotrophins and growth factors have been widely investigated for their ability to improve the efficacy of embryonic ventral mesencephalic grafts, parameters of the transplant procedure itself can also impact survival. Nikkah and colleagues demonstrated that decreasing the volume of cell suspension implanted, while holding the concentration of the cell suspension constant, led to an increase in the survival rate of grafted DA neurons (Nikkhah, et al., 1994). Given that optimal volume parameters were defined in the Nikkah study, we hypothesized that an optimal concentration may also exist. The current investigation examines the ability of cell suspension concentration to affect the survival rate and functionality of grafted THir neurons.

Methods

Unilateral Nigrostriatal Lesions

Thirty-two, male Fischer 344 rats (200-225g) were purchased from Harlan (Indianapolis, IN). All protocols utilized in this study were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center. Stereotaxic injections of 5μg of 6-hydroxdopamine hydrobromide (6-OHDA) per micro liter of 0.2% ascorbic acid-physiological saline solution were administered unilaterally into the medial forebrain bundle (AP −4.3, ML +1.2, DV −7.5) and the substantia nigra pars compacta (AP −4.8, ML +1.5, DV −7.5) at a rate of 1μl/min for two minutes. Nigrostriatal lesions were behaviorally verified by amphetamine-induced (5 mg/kg, i.p.) rotations. Rotational behavior was assessed by a computer assisted rotometer system that tabulated the number of complete ipsilateral and contralateral whole body turns in five minute intervals over an 85 minute period. Animals meeting the criteria of at least 6 ipsilateral rotations per minute were assigned to transplantation groups.

Cell Suspension

As previously described, tissue from the ventral mesencephalon was dissected from E14 Fischer 344 rat pups using sterile techniques and pooled in cold calcium-magnesium-free buffer (CMF) (Sortwell, et al., 2000). Cell suspensions were generated through a series of CMF rinses, incubated in trypsin, rinsed again in CMF, and triturated in 0.004% DNase to disperse the cells into solution. Trypan blue was added to a sample of the cell suspension that was viewed in a hemocytometer to assess cell viability and determine the cell count. Mesencephalic cells were diluted in neurobasal medium to the desired concentration. Each mesencephalon yielded approximately 392,000 cells following the cell suspension.

Transplant Procedures

Fischer 344 rats to be transplanted were divided into four groups. Group 1 (n=8) was grafted with ventral mesencephalic cells concentrated to 25,000 cells/μl. Group 2 (n=8) was grafted with mesencephalic cells concentrated to 50,000 cells/μl. Group 3 (n=7) was grafted with mesencephalic cells concentrated to 100,000 cells/μl. Group 4 (n=8) was grafted with

mesencephalic cells concentrated to 200,000 cells/μl. Cells were loaded into a 10μl Hamilton syringe with a 25-gauge needle attached to a stereotaxic needle holder for transplantation. Anesthetized rats (30 mg/kg, pentobarbital, ip) received two 0.5μl transplants at a rate of 0.5μl /min, unilaterally into the denervated striatum (AP +1.0, ML +2.5, DV −6.0; AP−0.6, ML+4.2, DV−6.8), of the cell suspension concentration specified for the assigned group. This cell suspension volume has been shown to optimize the survival of DA neurons transplanted into the rat striatum (Nikkhah, et al., 1994).

Graft Morphology

Animals from each group were sacrificed 10 days (n=22) and 6 (n=9) weeks post-transplantation. Rats were deeply anesthetized (60mg/kg, pentobarbital, ip) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PO_4 buffer (PFA). Brains were removed and post fixed for 24 hrs in 4% PFA and then transferred to 30% sucrose in 0.01 M PO_4 buffer. Brains were frozen on dry ice and 35Fm coronal sections were obtained using a sliding microtome. Every sixth section through the graft was analyzed for surviving tyrosine hydroxylase immunoreactive neurons (THir), as previously described (Marchionini, et al., 2004). Briefly, sections were incubated overnight at room temperature in antisera directed against TH (Chemicon, Temcula, CA, 1:4000). Following primary incubations and membrane permeabilization, sections were incubated in secondary antisera directed against mouse IgG (Chemicon, Temcula, CA, 1:400). In accordance with previous results demonstrating that THir cell death does not continue past four days post-transplantation (Sortwell, et al., 2000), quantification of THir neurons was performed on animals sacrificed 10 days post-transplantation. Absolute counts of THir neurons were made at 20x and the sum of these values was adjusted according to the method of Abercrombie (Abercrombie, 1946). Briefly, the total number of THir neurons in every sixth section throughout the entire rostral-caudal extent of the graft was estimated using the Abercrombie formula (N = A(M/L + M) where N is the average number of THir neurons per section, A is the crude count of THir neurons observed in the section, M is the thickness (in μ) of the section, and L the average length (in μ) of a THir soma). This value was then multiplied by 6 to estimate the number of THir neurons in each graft. The equations utilized to calculate the survival rate of THir neurons are shown in Figure 1 (Fawcett, et al., 1995). For quantification of soma size and THir staining intensity, three sections representing the core of the graft were evaluated at 20x magnification. Sample images taken from the corpus collosum served as a baseline value for THir staining intensity. All measurements were taken using the same light intensity and threshold settings, quantified with Sigma Scan Pro (San Rafael, CA) and represented as the total number of pixels/inch^2.

Statistical Analyses

Statistical comparisons of graft morphology between groups were analyzed with ANOVA, followed bya *post hoc* Fisher's PLSD test. The average THir soma size, staining intensity and grafted THir neuron number for each rat was calculated and compared between treatment groups. The number of amphetamine-induced rotations across experimental groups over time was analyzed with repeated-measures ANOVA.

Results

Behavioral Analysis

Prior to transplantation, there were no differences in rotational asymmetry between groups ($P>0.05$). Animals receiving transplants containing a greater total number of cells exhibited significantly greater recovery from amphetamine-induced rotational asymmetry [$F(3,12) =10.32$, $p<0.0001$](Figure 2.). Two weeks following transplantation, the 200,000 cells/μl group recovered to a greater extent that the 50,000 and 25,000 cells/μl groups ($P= 0.03$ and 0.02, respectively). Four weeks following transplantation the 200,000 and 100,000 cells/μl groups

both exhibited greater recovery from asymmetrical rotational behavior compared to the 50,000 cells/μl group (P=0.01). While not reaching statistical significance, this pattern persisted at 6 weeks post transplantation (P=0.055 and 0.074 respectively). Additionally, animals receiving transplants of 200,000 cells and 100,000 cells/μl recovered significantly from baseline measurements of rotational asymmetry (p= 0.007 and 0.002, respectively), while the groups receiving the 25,000 and 50,000 cells/μl did not.

Absolute Number of Surviving THir Neurons

Within treatment groups no differences in the number of grafted THir neurons were observed between the 10 day and 6 week timepoint, therefore for each group these two timepoints were combined for statistical analysis. Increasing the cell suspension concentration and in turn increasing the absolute number of cells transplanted, resulted in a proportional increase in the total number a THir neurons per graft [$F(3,23)=19.7$, $p<0.0001$] (Figure 3.). Grafts in the 200,000 cells/μl group had, on average, a greater number of THir neurons (524.61±78.96) than the 100,000 cells/μl (228.18±50.69, p=0.0003), 50,000 cells/μl (83.63±12.77, p<0.0001) and 25,000 cells/μl (33.60±4.67, p<0.0001) groups. Additionally, grafts in the 100,000 cells/μl contained significantly more THir neurons than those in the 25,000 cells/μl (p=0.02).

THir Neuron Survival Rate

Increasing the concentration of the mesencephalic cell suspension augmented the survival rate of transplanted THir neurons [$F(3,23)=3.33$, $p=0.037$]. As depicted in Figure 4, grafts of 200,000 cells/μl exhibited a significantly greater survival rate (5.5%±0.8) than those consisting of 50,000 and 25,000 cells/μl (3.4%±0.5, p=0.03 and 2.8%±0.4, p=0.02, respectively).

Soma Size and Immunoreactivity of THir Positive Neurons

No differences in soma size or pixel intensity of THir neurons were observed between the 25,000 cells/μl, 50,000 cells/μl, and 100,000 cells/μl groups, therefore these groups were combined for statistical analysis. Animals receiving grafts of more highly concentrated cell suspensions (200,000 cells/μl) displayed THir neurons with significantly larger soma size compared to grafts of lower concentrations [$F(1,18)=7.63$, $p=0.01$]. Additionally, analysis of average pixel intensity revealed that the 200,000 cells/μl group had a higher level of TH relative to all other groups [$F(1,18)=6.81$, $p=0.01$]. Figures 5 and 6 illustrate the impact of increased cell suspension concentration on the morphology of grafted DA neurons.

Discussion

Our results indicate that increasing the concentration of embryonic ventral mesencephalic cells prior to transplantation into the denervated striatum increases the survival rate and soma size of THir neurons. Specifically, cell suspensions of 200,000 cells/μl resulted in a significant survival effect when compared to 50,000 and 25,000 cells/μl. THir soma size, which has been shown to decrease in normal aging and MPTP treated non human primates, (Gerhardt GA, 2002), was also demonstrated to be augmented by increasing the cell suspension concentration prior to transplantation.

Previous *in vitro* studies have shown that cell suspension concentration influences the survival rate of other cell types (Fujita, et al., 2001; Fukushima, 1994; Sasaki, 1998). Sasaki et al., demonstrated that increasing the concentration of primary cortical neurons in culture increased survival rate and protected against serum withdrawal (Sasaki, 1998). Recent data from our laboratory demonstrated a similar effect in primary mesencephalic cultures (Marchionini, et al., 2003). While cell-cell interactions are likely contributing to this effect, there is evidence indicating that increasing cell density also results in the release of a survival factor(s) into the culture medium (Fujita, et al., 2001; Fukushima, 1994).

Trophic factor withdrawal has been suggested to be involved in the wave of cell death that occurs in ventral mesencephalic grafts during the four day period immediately following transplantation (Collier, et al., 1999; Sortwell, 2003). For trophic factors to have contributed to the increased survival rate and augmented morphology in the current study, they must be produced by cells located in developing ventral mesencephalon and have trophic effects on embryonic DA neurons. Mesencephalic type-1 astrocytes express GDNF during development; however, this has not been confirmed in the developmental stages corresponding to immediate post-grafting interval (E14-E18) (Schaar, et al., 1993). In the present study, grafts from higher cell suspension concentrations displayed characteristics similar to GDNF-treated embryonic ventral mesencephalic cultures and grafts (i.e. increased survival rate and soma size), indicating that GDNF could have influenced the current results (Collier and Sortwell, 1999; Granholm, et al., 1997; Rosenblad, et al., 1996). Another member of the TGFβ family, GDF5 may have contributed to the increase in survival seen in the current study. GDF5 peaks in the rat ventral mesencephalon at E14 and has been shown to increase DA neuron survival in E14 mesencephalic grafts (Sullivan, et al., 1998). PDGF has been shown to have trophic effects on DA neurons and is expressed in the E13-E15 ventral mesencephalon (Nikkhah G, 1993; Smits, et al., 1993). These studies imply that PDGF could have played a role in the increased survival rate seen in the present investigation. While the specific expression of BDNF in the ventral mesencephalon during prenatal development is not well established, it also is expressed in ventral mesencephalic grafts (Sautter, et al., 1998; Yurek and Seroogy, 2000). BDNF has been shown to increase graft-derived innervation of the host striatum without increasing survival (Yurek, et al., 1996); however, the inability of the current methods to evaluate graft-derived innervation makes it difficult to speculate on any role of BDNF in the current results.

Although the paracrine and autocrine affects of neurotrophic and survival factors may have contributed to the current results, another potential mechanism is the prevention of anoikis by an increase in cell-cell and extracellular matrix contact. Apoptosis resulting from detachment from the extracellular matrix and neighboring cells, termed anoikis, may take place during the dissociation of primary DA neurons prior to transplantation (Sortwell, 2003). A recent study from our laboratory, Marchionini et al, 2003, demonstrated that the addition of the extracellular matrix glycoprotein tenascin-C to cell cultures increased THir neuron survival. Furthermore, tenascin-C-treated low density grafts (3000 cells/μl) also displayed a higher survival rate. Tenascin-C also increased THir soma size in high density grafts (100,000 cells/μl) although no survival effect was observed (Marchionini, et al., 2003). The beneficial effects of tenascin-C on grafted embryonic DA neurons may have influenced the current data. Tenascin-C has been shown to be expressed and secreted by numerous populations of astrocytes (Mahler, et al., 1996; Meiners, et al., 1995). Although astrocytes have been shown to exert negative affects on neurons *in vitro* and in grafting paradigms, these studies utilized astrocytes in latter developmental stages (Krobert, et al., 1997; Meiners, et al., 1995). Also, it is likely that the mechanical dissociation involved in the present investigation resulted in isomorphic astrogliosis (Liberto, et al., 2004). This type of astrocyte activation results in the ability of astrocytes to increase many pro-survival processes. Among these processes may be the release of tenascin-C and other ECM proteins with positive modulatory affects. It is therefore possible that the release of these proteins contributed to the increased survival of grafted THir neurons in the present study. In addition to the release of these proteins, the ability of astrocytes to buffer ions and glutamate and contribute to glucose homeostasis may have increased cell survival in the present study.

In conclusion, increasing the concentration of the embryonic ventral mesencephalic cell suspension prior to transplantation into the denervated striatum augments the survival rate of THir neurons. Furthermore, increasing cell suspension resulted in larger soma size of THir neurons which may indicate an increase in the overall health of the grafts. The potential mechanisms involved in the current results include the paracrine and autocrine actions of

neurotrophic factors and the prevention of anoikis. While additional studies will be necessary to elucidate the exact mechanisms involved in the positive affects of increasing the cell suspension concentration prior to the transplantation of embryonic DA neurons, the current results highlight the importance of determining optimal parameters for transplantation of primary DA neurons in the striatum. With these parameters defined, methods can be standardized allowing for results from different laboratories to be accurately compared, expediting the refinement of this therapy in PD patients.

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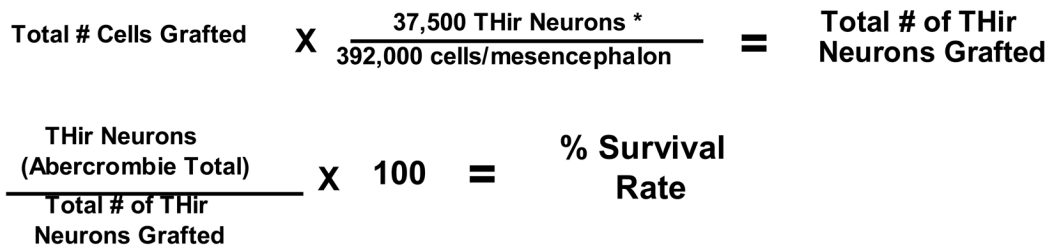

Figure 1. Calculation of THir neuron survival rates Following Transplantation

Survival Rate was calculated utilizing the following equations. *The number of THir neurons grafted was based on previous findings (Fawcett, et al., 1995).

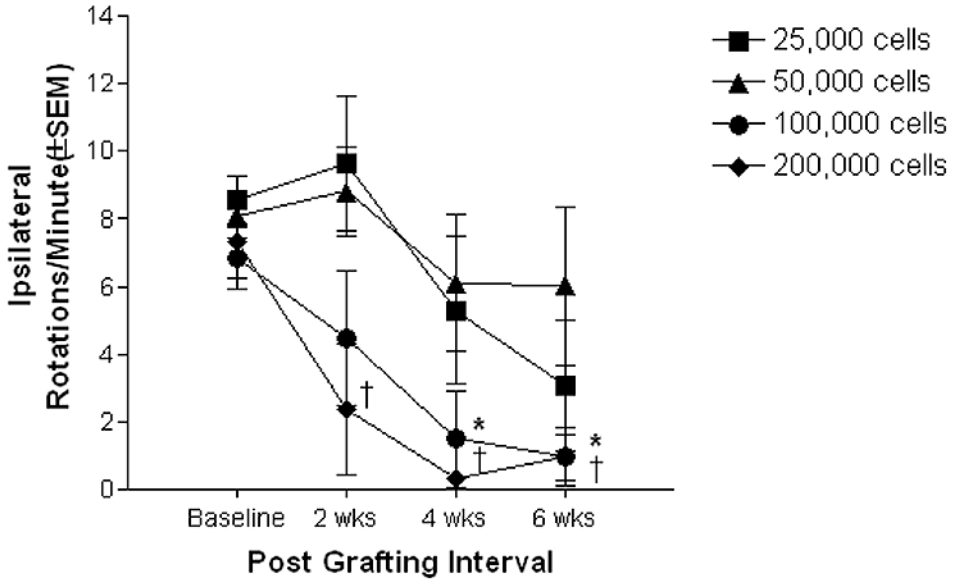

Figure 2. Effect of Cell Suspension Concentration on Amphetamine Induced Asymmetry

Five mg/kg of d-amphetamine was administered to rats unilaterally lesioned with 6-OHDA. Rats receiving grafts of 100,000 and 200,000 cells/μl displayed significant recovery from baseline rotational asymmetry (P=0.007 and P=0.002, respectively); * indicates significant recovery from baseline (200,000 cells/μl); †indicates significant recovery from baseline (100,000 cells/μl).

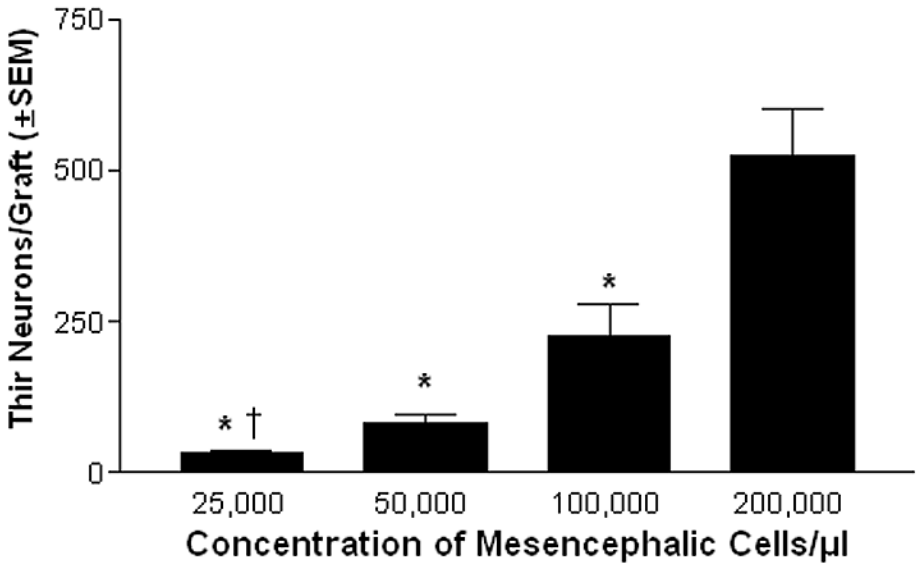

Figure 3. Effect of Cell Suspension Concentration on the Absolute Number of Surviving THir Neurons Following Transplantation

Primary DA neurons were concentrated to 25,000, 50,000, 100,000 or 200,000 cells/µl prior to transplantation into the denervated rat striatum. Increasing the concentration of cells per µl resulted in a dose dependent increase in the total number of THir neurons per primary mesencephalic graft. * indicates a significant difference between the 200,000 cells/µl group ($p \leq 0.05$); †indicates a significant difference between the 100,000 cells/µl group ($p \leq 0.05$).

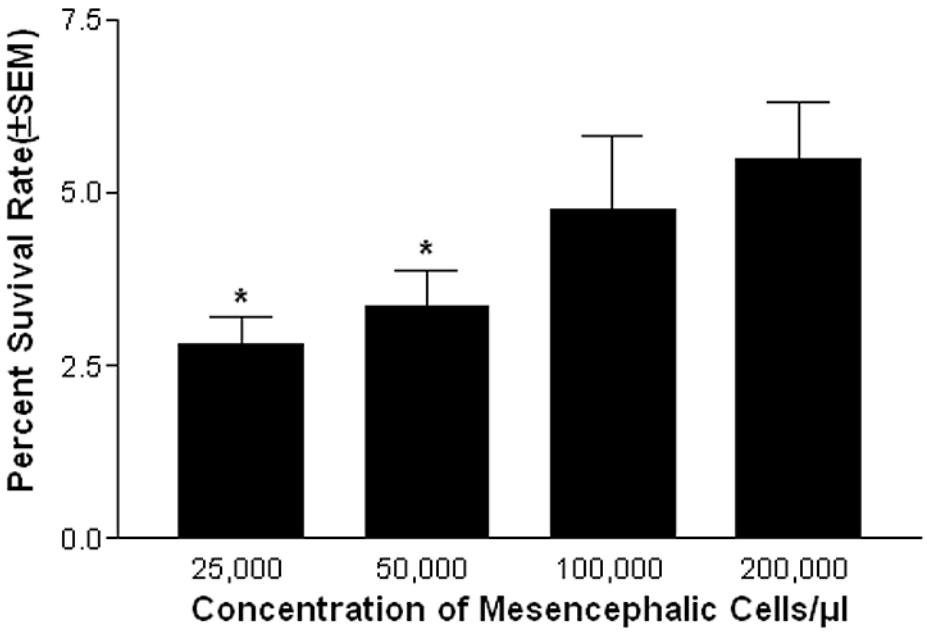

Figure 4. Effect of Cell Suspension Concentration on the Survival Rate of THir Neurons Following Transplantation

Primary DA neurons were concentrated to 25,000, 50,000, 100,000 or 200,000 cells/µl prior to transplantation into the denervated rat striatum. Increasing the concentration of cells per µl augmented the survival rate of THir neurons in primary mesencephalic grafts; *indicates a significant difference between the 200,000 cells/µl group ($p \leq 0.05$).

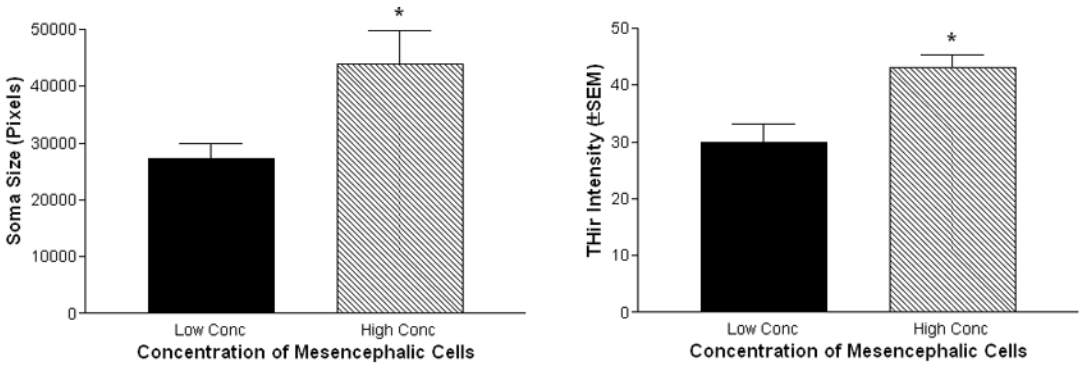

Figure 5. Effect of Cell Suspension Concentration on the Soma Size and Immunoreactivity of THir Neurons Following Transplantation

Primary DA neurons were concentrated to 25,000, 50,000, 100,000 or 200,000 cells/μl prior to transplantation into the denervated rat striatum. Grafts of more highly concentrated cell suspensions (200,000 cells/μl group; High Conc) displayed THir neurons with significantly larger soma size and TH staining intensity compared to grafts of lower concentrations (25,000, 50,000, and 100, 000 cells/μl groups; Low Conc). * indicates a significant difference between the High Concentration and Low Concentration groups (p=0.01).

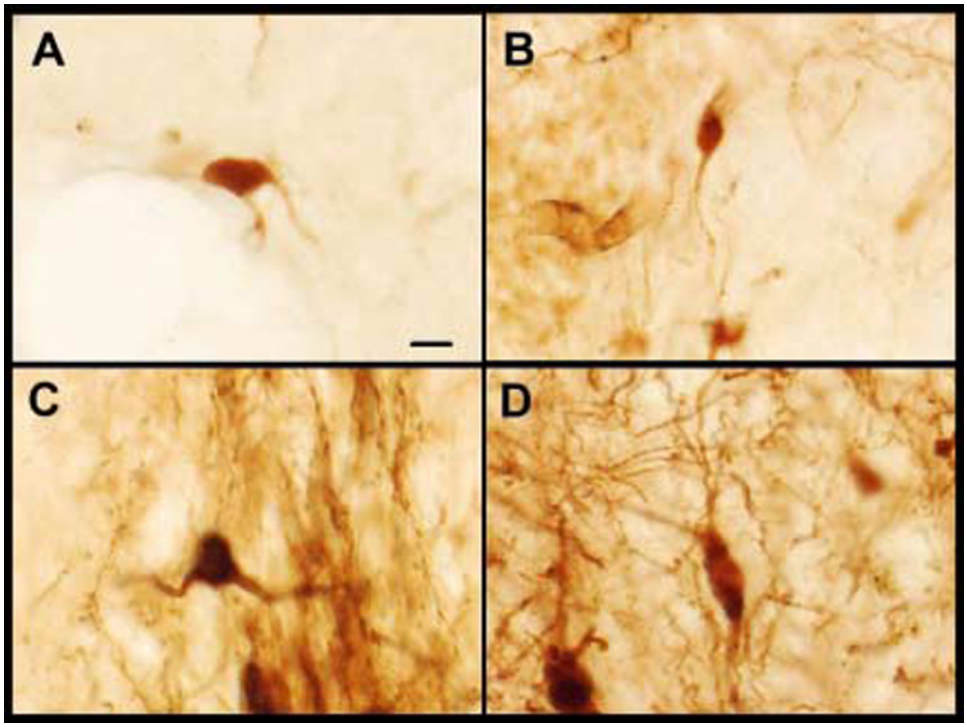

Figure 6. Effect of Cell Suspension Concentration on the Soma Size and Immunoreactivity of THir Neurons Following Transplantation

A. 25,000 cells/μl. B. 50,000 cells/μl. C. 100,000 cells/μl. D. 200,000 cells/μl. Scale bar in A. equals 10μm.