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Effects of Sertoli Cell Transplants in a 3-Nitropropionic Acid Model of Early Huntington's Disease: a Preliminary Study

ALBA I. RODRIGUEZ[b,d,*], ALISON E. WILLING[a,b,c], SAMUEL SAPORTA[a,b,c], DON F. CAMERON[e] and PAUL R. SANBERG[a,b,d]

[a]*Center of Excellence for Aging and Brain Repair,* [b]*Department of Neurosurgery,* [c]*The Neuroscience Program, College of Medicine, University of South Florida, Tampa, FL 33612, USA;* [d]*Department of Psychology, University of South Florida, Tampa, FL 33620, USA;* [e]*Department of Anatomy, College of Medicine, University of South Florida, Tampa, FL 33612, USA. albar@tampabay.rr.com, arodrig1@hfhs.org*

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Problems with immunosuppression and graft survival limit clinical applications of neurotransplantation protocols for neurodegenerative disease. Sertoli cells, testes-derived cells with immunosuppressive and trophic properties, may serve as an alternative cell source for transplantation. Sertoli cells were transplanted into the striatum of rats following two injections of 3-nitropropionic acid (3-NP) to determine whether they could ameliorate abnormalities in a model of early stage Huntington's disease. 3-NP-induced locomotor hyperactivity was significantly reduced in rats receiving Sertoli transplants compared to controls, with some behaviors returning to baseline. Sertoli cells survived in the striatum without systemic immunosuppression and some formed tubule-like structures. These results show that Sertoli transplants are able to ameliorate locomotor abnormalities in a 3-NP model of early HD. Thus, Sertoli cells should be further evaluated as a possible treatment strategy for the early stages of Huntington's disease.

Keywords: Sertoli cells; Neurotransplantation; Striatum; Behavior; Huntington's disease

INTRODUCTION

Huntington's disease (HD), an age-dependent inherited disorder, is a debilitating, and eventually fatal neurodegenerative disease with characteristic cognitive and motor symptoms that eventually progress to incapacitating extremes. Although recent research has led to the isolation of the HD gene (The Huntington's Disease Collaborative Group, 1993) and the characterization of the pathophysiology of the disorder, there is as yet no treatment that reverses or slows the neurodegeneration and progression of the disease.

The 3-nitropropionic acid (3-NP)-treated rat provides a good model in which to examine possible treatments for HD, as it closely emulates many of the neuropathological and behavioral features of HD and may have a similar mechanism of action (Alexi *et al.*, 1998). Administration of 3-NP produces selective lesions of the striatum (Beal *et al.*, 1993; Brouillet *et al.*, 1993) as well as cognitive and motor alterations (Borlongan *et al.*, 1995a,b; Duckworth *et al.*, 1999). More importantly, the 3-NP model is able to replicate the progressive nature of HD. Repeated administration at low doses (Borlongan *et al.*, 1997b) causes progressive motor alteration from hyperactivity (following 2 injections of 3-NP) to hypoactivity (following 4 injections of 3-NP) that may parallel the progression from chorea to a Parkinsonian-like syndrome seen in the human disorder. This progression allows for the examination of the neuropathological and behavioral features at different stages of the disease.

While neural transplantation therapy provides promise as an alternative treatment protocol for neurodegenerative disorders such as Parkinson's disease (PD) and HD (Bjorklund *et al.*, 1980; Dunnett *et al.*, 1981; Koutouzis *et al.*, 1994; Borlongan *et al.*, 1995a), ethical concerns inherent with the use of human fetal tissue, problems with graft survival and the need for long term immunosuppression limit widespread use of human fetal tissue neurotransplantation (Berden *et al.*, 1985; de Groen *et al.*, 1987; Famiglio *et al.*, 1989; Sauer and Brundin, 1991; Nakao *et al.*, 1994;

*Corresponding author, current address: Henry Ford Health System, William T. Gossett Neurology Laboratories, 1 Ford Place 4D, Detroit, MI 48202. Tel: +1-313-874-6954; Fax: +1-206-350-4778; Email: albar@tampabay.rr.com or arodrig1@hfhs.org
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Kordower *et al.*, 1998). These limitations have lead researchers to investigate methods for increasing the survival of grafted cells through better immune suppression and trophic support. (Granholm *et al.*, 1997; Zawada *et al.*, 1998; Armstrong and Svendsen, 2000; Armstrong *et al.*, 2000; Fink *et al.*, 2000).

Recent studies suggest that Sertoli cells (SCs) may alleviate the problems of both immunosuppression and graft survival in neural transplantation. SCs are found in the seminiferous tubules of the testis where they provide trophic and immune support to the developing germ cells. SCs secrete numerous immunosuppressant and trophic factors including Fas (CD95) ligand (FasL), transforming growth factors (TGF-α and -β), interleukin 1α (IL-1α) and interleukin 6 (IL-6), platelet-derived growth factor (PDGF), and neurturin (NTN) (Hunt and Eardley, 1986; Griswold, 1993; Skinner, 1993; Gnessi *et al.*, 1995; French *et al.*, 1996; Cudicini *et al.*, 1997; Sanberg *et al.*, 1997b; Widenfalk *et al.*, 1997; Piccirillo *et al.*, 1998). Co-transplanted SCs are able to provide localized long-term immunosuppression for grafted islet tissue (Selawry and Cameron, 1993) and human neuron-like (hNT) cells (Willing *et al.*, 1999); and rat and porcine Sertoli cells are able to survive in the rat striatum for at least two months without the use of immunosuppressant drugs (Saporta *et al.*, 1997). These trophic capabilities may explain why SCs transplanted alone into the striatum of hemiparkinsonian rats are able to produce behavioral recovery (Borlongan *et al.*, 1997a; Sanberg *et al.*, 1997a).

Sertoli cells provide a cocktail of trophic factors following transplantation into the brain, and can ameliorate behavioral deficits in a rat model of PD, suggesting that Sertoli cell transplants may provide a novel therapeutic approach to treatment of other neurodegenerative disorders. In order to further examine whether SCs can be considered for clinical use, we recently conducted a study to ensure that the cells themselves produce no adverse effects when transplanted into the central nervous system (Rodriguez, 2002; Rodriguez *et al.*, 2002). We assessed the behavioral effects of transplanting porcine Sertoli cells into the striatum of normal rats. Although there were significant increases in nocturnal locomotor activity over time following both sham and Sertoli transplants, Sertoli animals exhibited less behavioral alteration than sham controls. Histological examination of the striatum demonstrated surviving Sertoli cell transplants in an intact striatum. These results indicated that Sertoli cell xenografts may be a safe alternative cell source for neurotransplantation procedures requiring immune or trophic support.

The present study was conducted to extend these findings into a 3-NP animal model of HD. In this randomized study, Sertoli cells were transplanted into the striatum of rats following a short course of 3-NP treatment in order to determine whether they were able to ameliorate any of the behavioral and neuropathological abnormalities seen in a 3-NP model of the early stage of Huntington's disease.

METHODS

Subjects

Adult, male Sprague-Dawley rats (n = 16), approximately 16 weeks of age, were housed in pairs in a room with controlled temperature and humidity on a 12-h light-dark cycle. The animals had free access to food and water while in their home cages. Animal care and handling were conducted in accordance with the National Institutes of Health guidelines for animal treatment. An additional group of rats (n = 8) that did not receive 3-NP, but that were otherwise treated in the same fashion, were used as a comparison for evaluation of the effects of 3-NP on ventricle size; behavioral data from this group was reported in Rodriguez *et al.*, 2002.

Procedures

Rats received a short course of 3-NP as described fully in Borlongan *et al.* (1997b) to simulate the early stage of HD. 3-NP (Sigma, in 0.9% sterile saline, pH 7.4) was administered at 10 mg/kg i.p., every fourth day to reach sustained hyperactivity (2 administrations).

Two weeks after the final 3-NP administration, rats were randomly divided into two groups and received bilateral transplants into the striatum (bregma + 1.2 AP, ± 3.4 ML, and - 4.2, 4.7 DV) deposited into two sites per side (2 µl per site). Surgical procedures were carried out under sterile conditions. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and placed into a Kopf stereotaxic frame. The control (sham operated) group (n = 8) received media only (15 mM HEPES in Hank's Balanced Salt Solution (HBSS), while the Sertoli group (n = 8) received rat SCs in the same media. Rat SCs were isolated from 16-19-day old male Sprague-Dawley rats (Harlan Sprague Dawley, Inc.) according to the procedure used by Selawry and Cameron (1993) and described briefly as follows: Rats were anesthetized with Equithesin; testes were removed from the testicular capsule and subjected to sequential enzymatic treatment at 37°C using 0.25 % trypsin and 0.2% collagenase. Resulting Sertoli aggregates were plated and incubated at 37°C in 5% CO_2-

95% air for 48 h. They were then harvested and labeled with a fluorescent lipophilic carbocyanine dye, DiI (1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), washed 3 times, and spun down at 700 RPM for 3 minutes. The supernatant was removed and they were resuspended in 100 µl media. Trypan blue dye exclusion was used to assess number of cells per µl as follows: 1 µl of cells was combined with 9 µl of Trypan blue solution and the number of living cells and labeled dead cells were counted to obtain the percent viability. Cell numbers were adjusted to approximately 20,000 per µl with Hank's Balanced Salt Solution (HBSS; Gibco BRL) plus HEPES for transplantation.

Behavioral Analyses

Locomotor activity was assessed at several time points: prior to any treatment to establish a baseline, after the final 3-NP administration to examine 3-NP-induced behavioral alterations, and at 4-, and 12-weeks post-transplant to examine recovery. Following injection with either 3-NP (at the second 3-NP test time) or saline (at baseline and post-transplant test-times), rats were placed individually into the Digiscan-16 Animal Activity Monitors (Omnitech Electronics, Inc., Columbus, OH, USA) during the dark phase of a 12-h light/dark cycle, starting 1 h before lights out (approximately 5:00 pm - 6:00 am) with food and water freely available. Baseline locomotor activity was obtained using the second of two pretests (the first was used to allow rats to habituate to the Digiscan monitor). Activity measures during peak activity times (approximately 7:00 pm-1:00 am) were used for statistical analyses. An examination of the data showed three types of behaviors that were most affected by 3-NP: 1) Ambulatory activity was comprised of horizontal activity (HA, total number of beam interruptions that occurred in the horizontal sensor) horizontal movement time (HT, amount of time the animal was ambulating), and number of horizontal movements (HM, number of discrete horizontal movements); 2) Rearing activity was comprised of vertical activity (VA, total number of beam interruptions that occurred in the vertical sensor), vertical time (VT, amount of time the animal was rearing), and number of vertical movements (VM, number of discrete vertical movements); 3) Stereotypic activity was comprised of stereotypy count (SC, number of beam breaks that occurred during periods of stereotypic activity), stereotypy time (ST, total amount of time stereotypic behavior was exhibited), and number of stereotypical movements (SM, number of episodes of stereotypic behavior).

To avoid inflating the type 1 error rate in conducting separate analysis on each of the three related behaviors in each category, a composite score was obtained for each type of behavior (ambulatory, rearing and stereotypic) by averaging three individual measures. Because the individual behaviors do not use the same scale, the data were standardized as a percent of the baseline measure prior to averaging for a composite score. For example the composite score for ambulatory activity at a given test time is calculated as:

Ambulatory activity = [% baseline HA + % baseline HT + % baseline HM]/3

A 2-way mixed model Analysis of Variance (ANOVA) was conducted for each of the composite measures to examine the effects of transplant type (Sertoli vs. Media) on behavior across the different testing times with post-hoc Tukey's tests.

Histology and Immunohistochemistry

Following the last behavioral test, animals were perfused with 50-100 ml of normal saline followed by 400-500 ml of 4% paraformaldehyde, and brains were removed, post-fixed, cryopreserved in 10% sucrose in phosphate buffered saline (PBS) for one day, transferred to 20% sucrose in PBS, frozen, and sectioned at 30 µm. Alternate brain sections were then evaluated with the following:

1) Immunofluorescent photomicroscopy of DiI-labeled Sertoli cells to evaluate location and survival of Sertoli cells;

2) Nissl staining with cresyl violet to examine morphology of the striatum and transplant site;

3) Light photomicroscopy to evaluate ventricle size, as a measure of global striatal damage/atrophy. Thaw-mounted sections of the brain between bregma 1.00 mm and bregma 0.2 mm (Paxinos and Watson, 1986) were photographed at 1.5x magnification with an Optronix digital camera using Magnafire software. Ventricle size was measured using Sigma Scan Pro 5.0. Resulting data were analyzed statistically using independent *t*-tests to evaluate differences in ventricle size between control and Sertoli groups. Additionally, ventricle sizes of control rats receiving 3-NP injections in this experiment were compared to a group of rats not receiving 3-NP ($n = 8$); and

4) Tyrosine hydroxylase (TH) (1:2000, Diasorin) to evaluate damage to dopaminergic fibers in the striatum. Sections from each brain were permeabilized for 1 h at room temperature with 0.03% Triton X-100 (Sigma, St. Louis, MO, USA), 0.03% Lysine (Sigma), and 10%

normal horse serum (Vector Laboratories, Burlingame, CA, USA), washed with PBS, and incubated with the primary antibody overnight at 4°C in a humidified chamber. They were then washed and incubated for 1 h at room temperature with a biotinylated horse anti-mouse secondary antibody (1:200, Vector), washed and incubated with the ABC system (Vectastain ABC Elite Kit, Vector) for 1 h, then washed again. Bound antibody was visualized with 3.3'-diaminobenzidine (DAB, Pierce, Rockford, IL, USA) as the chromogen. Following immunohistochemistry, sections were examined and photographed on a Leitz Orthoplan microscope, and qualitative analyses were conducted.

RESULTS

Behavioral Analyses

Figure 1 shows levels of ambulatory activity (A), rearing activity (B), and stereotypic activity (C) for control and Sertoli animals following a short course of 3-NP administration and at 4- and 12-weeks post-transplant. One rat in the control group and one in the Sertoli group died during the experiment and were thus excluded from the behavioral analyses. The data were evaluated to assure that they met assumptions of statistical analyses. Due to the small sample size and heterogeneity of variance, data were examined for outliers using objective criteria based on distance from the mean. If data points fell outside this range, they were excluded; these criteria were applied equally to all groups. This resulted in decreased variability, while retaining the same pattern of results seen in the original data.

Following the second injection of 3-NP, control and Sertoli rats were hyperactive, with ambulatory activity levels at 158% and 140% of baseline, respectively. Ambulatory activity at 4-weeks post-transplant increased in controls to 174% but decreased in Sertoli rats to 121% of baseline. By 12-weeks, behavior of Sertoli rats had decreased to 107% of baseline, while that of controls remained high at 165% of baseline (FIG. 1A). Statistical analyses showed a significant effect of transplant type [$F(1,12) = 5.40$, $p<0.04$] but no significant time [$F(2,24) = 0.63$, $p>0.53$] or interaction effects [$F(2,24) = 1.34$, $p>0.28$].

Rearing activity levels (FIG. 1B) were above baseline for both groups of rats at all treatment times tested. However, those of Sertoli rats were less increased overall than those of controls, especially at 4 weeks (controls: 245%, Sertoli: 181%) and 12 weeks (controls: 302%, Sertoli: 178%). There was no significant effect

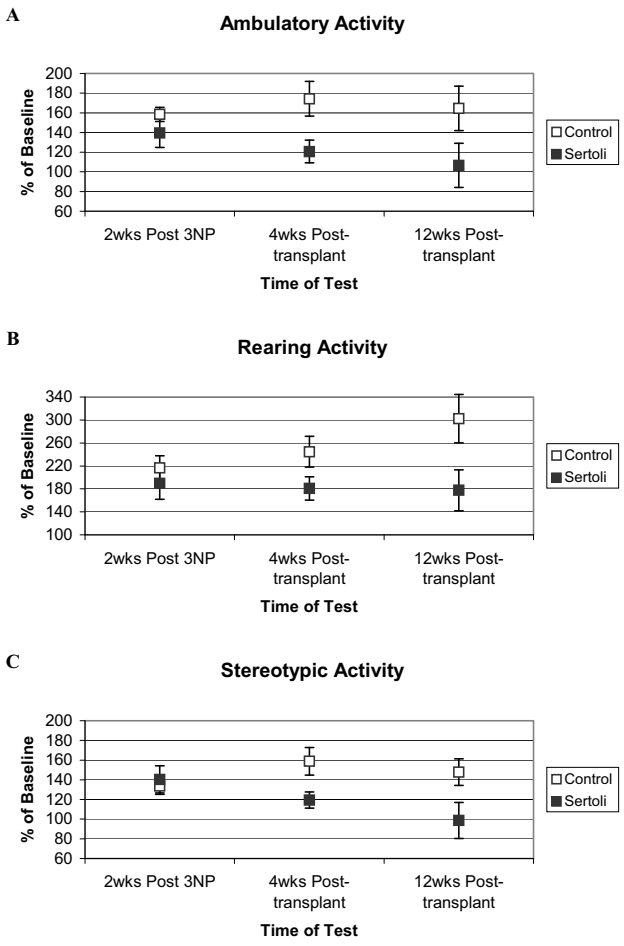

FIGURE 1 Effects of Sertoli cell transplants on Digiscan activity measures. Ambulatory (**A**), rearing (**B**), and stereotypic (**C**) activity means ± SEM following 3-NP and at 4 and 12 weeks post-transplant. Control: $n = 7$, Sertoli: $n = 7$.

of transplant type [$F(1,12) = 3.68$, $p>0.07$] or time [$F(2,24) = 2.44$, $p>0.10$]. However, the transplant type by time interaction was significant [$F(2,24) = 4.08$, $p<0.03$], indicating that the change in activity over time was different for the two groups. Post-hoc tests showed no differences between the control and Sertoli groups following 3-NP treatment ($p>0.54$) or at 4 weeks post-transplant ($p>0.14$). However, by 12 weeks post-transplant, Sertoli rats were significantly less hyperactive than controls ($p<0.009$).

Stereotypic activity levels (FIG. 1C) following 3-NP treatment were 134% of baseline for controls and 140% for Sertoli. However, the activity of rats with Sertoli transplants decreased to baseline levels (99%) by 12 weeks post-transplant, while those of controls remained high (148%). The effects of transplant type [$F(1,12) = 3.02$, $p>0.10$] and time [$F(2,24) = 1.95$, $p>0.16$] were not significant. However, there was a significant interaction effect ($p<0.009$), indicating that the rate of change in stereotypic activity across time was different for the two groups. Post-hoc tests showed no

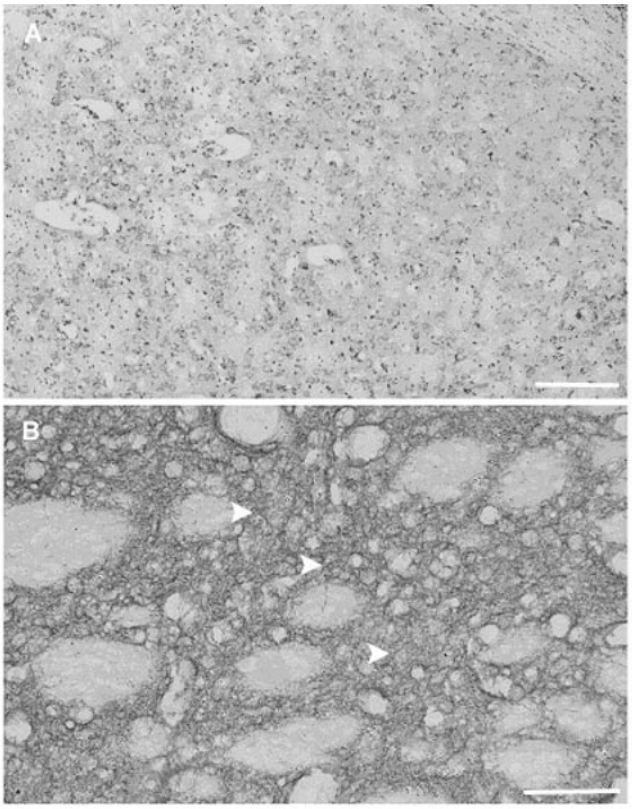

FIGURE 2 Photomicrographs of the striatum of a 3-NP treated rat. **A**) Nissl-stained striatum shows no obvious lesions (scale bar = 200 μm). **B**) Striatum processed with TH immunohistochemistry shows no obvious damage; the TH-positive fibers form a complicated network throughout the striatum. Selected fibers are indicated with arrowheads. (scale bar = 100 μm).

significant difference between control and Sertoli rats following 3-NP administration ($p>0.72$), and significant differences at both 4 weeks ($p<0.05$) and 12 weeks ($p<0.02$) post-transplant, with Sertoli rats exhibiting less hyperactivity and actually returning to baseline levels by 12 weeks.

Histology and Immunohistochemistry

Examination of the striatum for 3-NP-induced damage showed no obvious lesions in any of the animals. Figure 2 shows representative sections from a control rat that received 3-NP. Nissl-stained neurons were dispersed throughout the striatum (FIG. 2A), and TH-reactive fibers were evident (FIG. 2B). There did not appear to be any visible damage to the striatum following this 3-NP dosing regimen. Nevertheless, an analysis of ventricle size showed that control rats treated with 3-NP had larger ventricles compared to rats that were not treated with 3-NP, indicating 3-NP-induced striatal atrophy (FIG. 3). Rats treated with 3-NP and given Sertoli transplants had a tendency for smaller ventricles than control 3-NP rats (FIG. 3).

The injection/transplantation sites were clearly visible in the Nissl-stained striatum of control and Sertoli animals (FIGs. 4A and 4B), and the structural organization of the host striatum was not disturbed by the Sertoli cell transplant (FIG. 4B). Furthermore, tubule-like structures (FIGs. 4B and 4C) had formed within the Sertoli transplant site in some animals; these structures were not observed in the sham-operated controls (FIG. 4A). Examination of adjacent sections under epifluorescence showed that these tubules are formed from DiI-labeled SCs (FIG. 4D).

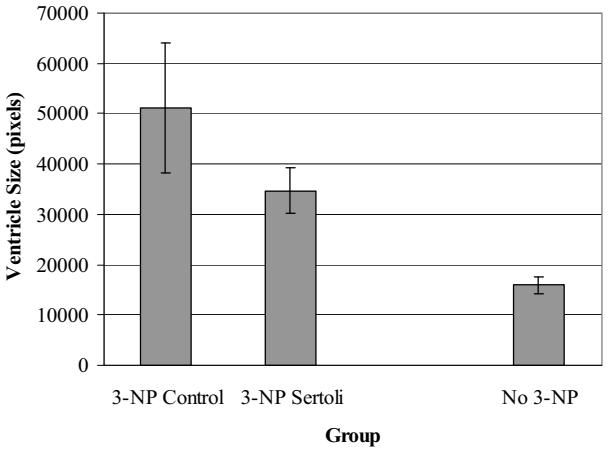

FIGURE 3 Ventricle size of rats treated with 3-NP. Rats that were injected with 3-NP and then had control (sham transplants) had larger ventricles than 3-NP treated animals with Sertoli cell transplants. For comparison, ventricle size in non-3-NP treated animals was smaller. Values indicate mean number of pixels ± sem.

DISCUSSION

This small preliminary study evaluated the restorative effects of Sertoli cell transplants at the early stage of the 3-NP model of HD. Animals were given a short-course of 3-NP administration to simulate early HD, and the behavioral effects of Sertoli cell transplants were evaluated through 12-weeks post-transplant. Interestingly, there was a large amount of behavioral variability in these animals. As a consequence of this variability, the data were normalized to assist in the statistical analysis. Nevertheless, there was a marked tendency in the behavioral effects of the 3-NP administration with or without Sertoli cell transplantation that was not changed by data normalization. Further, these data are consistent with recent evidence indicating that the most prevalently used rat strain in 3-NP studies, the Sprague-Dawley, exhibits great inter-animal variability in response to chronic 3-NP treatment. With chronic low-dose treatments there is generally substantial mortality (10-20%) early in the treatment course and only 30-40% exhibit dorsolateral striatal lesions; and acute

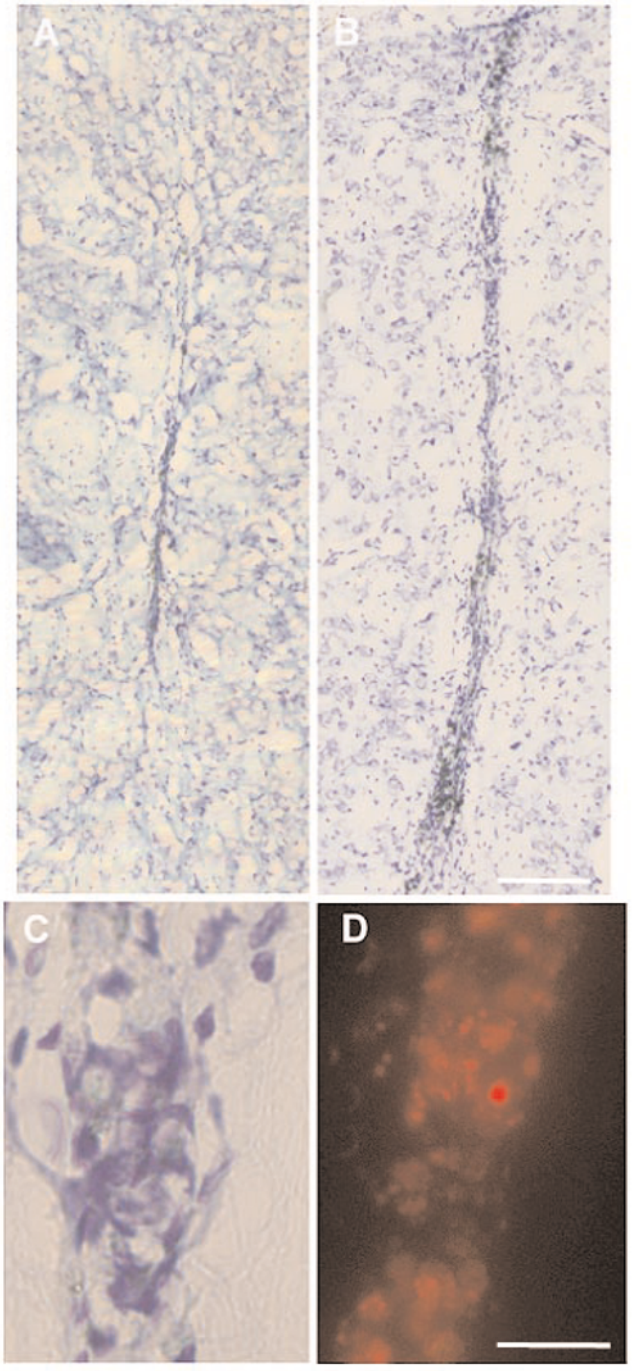

FIGURE 4 Photomicrographs of the graft site in a Control and Sertoli-transplanted rat striatum. **A**) Nissl-stained media-injection site in the striatum. **B**) Transplant site in a section from a Sertoli-transplanted animal. The transplanted cells have aligned themselves in the host striatum. **C**) Higher magnification view of the transplant in B. **D**) Epifluorescence image from an adjacent section showing DiI-labeled SCs. Scale bar in **A, B** = 200 μm. Scale bar in **C, D** = 50 μm.

higher-dose administration leads to even higher mortality (Ouary *et al.*, 2000). This may partially explain the variability in behavioral response seen in the present study. In fact, recent studies show more consistent behavioral and neuropathological effects of 3-NP using Lewis rats (Ouary *et al.*, 2000; Blum *et al.*, 2001). Future studies will employ this model.

As in previous studies (Borlongan *et al.*, 1995c; 1997b), 3-NP treatment produced locomotor hyperactivity. This hyperactivity was significantly reduced in rats receiving Sertoli transplants as compared to those receiving control transplants. In fact, by 12 weeks post-transplant Ambulatory and Stereotypic activity of Sertoli-transplanted rats had returned to baseline levels, while those of control-transplanted rats remained high.

An examination of the brains of the control 3-NP-treated animals showed no obvious striatal lesions or loss of TH fibers. Future studies should more closely evaluate the effects of this low-dose 3-NP regimen on the striatum using more sensitive measures (e.g. biochemical analysis of TH activity or quantification of TH-reactive fibers). Although there was no visible damage to striatum in this study, there was evidence of striatal atrophy, seen as a significant increase in ventricular size in controls that received 3-NP as opposed to rats that did not. There was a nonsignificant tendency for less striatal atrophy in rats that received Sertoli transplants. SCs survived in the striatum without systemic immunosuppression, and in some cases, may have been able to reorganize from isolated cells or clusters (as transplanted) to form tubule-like structures. Thus, the extent of survival and organization of transplanted SCs may also have affected any behavioral and neuropathological recovery.

In conclusion, the results of this experiment show that Sertoli transplants are able to ameliorate locomotor abnormalities in a 3-NP model of early HD. Furthermore, we showed in a previous study (Rodriguez *et al.*, 2002) that Sertoli xenografts also survive in the normal host brain without immunosuppression and produce fewer alterations than control transplants. Thus, Sertoli cells may provide a safe, useful alternative cell source for neurotransplantation protocols for neurodegenerative diseases such as HD. Future studies should further evaluate these cells as a possible treatment strategy for the early stages of HD and develop methods of ensuring consistent numbers of transplanted cells and evaluating survival of the Sertoli cells at post-transplant times.

Disclosures

PRS and DFC are co-founders of Saneron CCEL Therapeutics and SS and AEW are consultants.

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