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Transplantation of Human Retinal Pigment Epithelial Cells in the Nucleus Accumbens of cocaine self-administering rats provides protection from reinstatement

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Abstract

Chronic exposure to drugs and alcohol leads to damage to dopaminergic neurons and their projections in the ‘reward pathway’ that originate in the ventral tegmental area (VTA) and terminate in the nucleus accumbens (NAc). This damage is thought to contribute to the signature symptom of addiction: chronic relapse. In this study we show that bilateral transplants of human retinal pigment epithelial cells (RPECs), a cell mediated dopaminergic and trophic neuromodulator, into the medial shell of the NAc, rescue rats with a history of high rates of cocaine self-administration from drug-seeking when returned, after 2 weeks of abstinence, to the drug-associated chamber under extinction conditions (i.e., with no drug available). Excellent survival was noted for the transplant of RPECs in the shell and/or the core of the NAc bilaterally in all rats that showed behavioral recovery from cocaine seeking. Design based unbiased stereology of tyrosine hydroxylase (TH) positive cell bodies in the VTA showed better preservation ($p < 0.035$) in transplanted animals compared to control animals. This experiment shows that RPEC grafts provide beneficial effects to prevent chronic relapse in drug addiction via its effects directly on the NAc and its neural network with the VTA.

Keywords

RPEC; cocaine; addiction; reinstatement; transplant; rat

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1. INTRODUCTION

Addiction is a major disorder in which chronic exposure to alcohol or drug results in damage of the mesolimbic dopaminergic pathway projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (Trulson et al., 1987), and this damage is thought to contribute to the signature symptom of addiction: chronic relapse (reviewed in (Feltenstein and See, 2008; Pereira et al., 2015)). Recent studies have shown that the VTA derived dopaminergic inputs into the NAc have importance to the pathophysiology of reinstatement of addictive behavior (Shen et al., 2014; Smith and Villalba, 2013; Villalba and Smith, 2013). A subset of patients with Parkinson's disease (PD) also develop "dopamine dysregulation syndrome" which shares phenomenological similarities to behavior noted in chronic drug addiction such as punting, gambling, hypersexuality, impulsivity and reward seeking behavior (Zurowski and O'Brien, 2015). The clinical symptoms of dopamine dysregulation syndrome that occur in PD patients can be mitigated by replacing intermittent high dose dopaminergic stimulation that is used for motor control with continuous low-dose dopaminergic stimulation (Catalan et al., 2013; Samuel et al., 2015). Moreover, dopaminergic medications have been shown to gain psychostimulant-like properties in PD and such psychostimulant-like effects can be treated with deep brain stimulation surgery (DBS) or by regularizing continuous dopaminergic stimulation in PD patients as closely as possible to physiological levels (Catalan et al., 2013; Devoto et al., 2014; Engeln et al., 2013; Samuel et al., 2015; Schmitz et al., 2014; Zurowski and O'Brien, 2015). These similarities between drug addiction and PD suggest that normalizing dopamine dysregulation using a cell transplant that provides continuous dopamine replenishment and/or may prevent drug addiction induced neuropathology, may have therapeutic benefit against reinstatement of addictive behaviors.

Cell transplantation experiments for dopamine replacement and for trophic repair of degenerating dopaminergic pathways have been extensively employed for PD with some level of success. Indeed, clinical dopaminergic cell transplantation studies have been revived after a period of clinical moratorium and now such transplanted patients are being longitudinally followed in a registry (Abbott, 2014; Subramanian and Deogaonkar, 2013). Anatomically, the dopaminergic 'reward pathway' parallels the dopaminergic motor pathway. Yet, in comparison to the myriad of studies designed to treat parkinsonian symptoms with dopaminergic transplants of one sort or another (reviewed in (Petit et al., 2014)), we are not aware of any dopaminergic cell transplantation experiments to 'treat' the chronic disease of addiction. In this study, we tested whether a NAc graft of fetal human retinal pigment epithelial cells (RPEC), could prevent cue-induced reinstatement following abstinence in a rat model of cocaine addiction. RPECs secrete L-dopa, the precursor to dopamine, glial derived neurotrophic factor (GDNF), the most potent trophic factor for dopaminergic neurons, pigment epithelium derived growth factor (PEDF), a serpin that has trophic effects on dopaminergic neurons, and several anti-inflammatory cytokines that prevent immune rejection and barrier protection to the retina (Adjianto and Philp, 2014; Chiba, 2014; McKay et al., 2006; Ming and Le, 2007; Ming et al., 2009; Sparrow et al., 2010; Tombran-Tink and Barnstable, 2003). RPECs have been extensively tested as cell transplants in the eye for macular degeneration and in the brain for PD. These transplants

have been tolerated well in clinical trials when the tissue source was of fetal or embryonic origin (Chiba, 2014). RPEC transplants have survived across species xenotransplantation without the need for chronic immunosuppression and have been imaged post transplantation to track their survival in vivo (Cepeda et al., 2007; McKay et al., 2006; Ming and Le, 2007; Ming et al., 2009; Stover et al., 2005; Stover and Watts, 2008; Subramanian et al., 2002). This is a potential major advantage to using RPECs for cell transplants as they can immunomodulate the graft site via cytokines that they secrete. In contrast, RPEC grafts derived from post-natal donors have had poor survival and have failed in clinical trials (Farag et al., 2009; Gross et al., 2011). Therefore, we tested the effects of bilateral NAC transplants of fetal origin RPECs on cue-induced reinstatement of cocaine seeking behavior in a rat model of drug self-administration. Outcomes were assessed using behavioral tests for cocaine reinstatement, histological evidence for RPEC graft survival within the NAc, host dopaminergic neuronal survival in the VTA estimated using stereology of TH positive neurons (TH is the rate limiting enzyme for dopamine synthesis), and for dopa decarboxylase (DDC, the enzyme involved in converting L-dopa to dopamine).

2. METHODS

2.1 Subjects

All procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee and animals were housed in AALAC accredited animal facility with full time veterinary care compliant with the latest NIH guidelines for humane and ethical treatment of animals. This study was conducted in three replications. The subjects were 126 (n = 45 for Replication 1, n = 34 for Replication 2, and n = 47 for Replication 3) naïve, male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC), approximately three months of age at the beginning of the experiment. Because of complications during surgeries, loss of catheter patency, unexpected death, and poorly placed beads (for the vehicle animals or RPEC grafts) the final number of subjects was 84 (n = 32 from Replication 1, n = 22 from Replication 2, and n = 30 from Replication 3). Rats were housed individually in standard wire mesh cages, in a colony room with temperature, humidity, and ventilation controlled automatically. They were maintained on a 12/12 h light/dark cycle, with lights on at 0700h. All testing was conducted during the light phase of the cycle. The rats were allowed ad lib access to food (Harlan Teklad, Madison, WI) and water, except where otherwise noted.

2.2 Catheter Construction and Surgical Procedures

Self-administration catheter. Intra-jugular catheters were custom-made in our laboratory as described by Grigson and Twining (Grigson and Twining, 2002) and Twining et al. (Twining et al., 2009). Catheter implantation. Rats were anesthetized using an intraperitoneal (i.p.) injection of 70 mg/kg ketamine/10 mg/kg xylazine, and catheters were implanted into the jugular vein as described by Twining et al. (Twining et al., 2009). After surgery, rats were allowed at least one week to recover. General maintenance of catheter patency involved daily examination and flushing of catheters with heparinized saline (0.2 ml of 30 IU/ml heparin). Catheter patency was verified, as needed, using 0.2 ml of propofol (Diprivan 1%) administered intravenously.

2.3 Apparatus

Each rat was trained in one of twelve identical operant chambers (MED Associates, St. Albans, VT) described by Grigson and Twining (Grigson and Twining, 2002) and Twining et al. (Twining et al., 2009). Each chamber measured 30.5 cm in length $\times$ 24.0 cm in width $\times$ 29.0 cm in height, and was individually housed in a light- and sound-attenuated cubicle. The chambers consisted of a clear Plexiglas top, front, and back wall. The side walls were made of aluminum. Grid floors consisted of nineteen 4.8-mm stainless steel rods, spaced 1.6 cm apart (center to center). Each chamber was equipped with two retractable sipper spouts that entered through 1.3-cm diameter holes, spaced 16.4 cm apart (center to center). A stimulus light was located 6.0 cm above each spout. Each chamber was also equipped with a houselight (25 W), a tone generator (Sonalert Time Generator, 2900 Hz), and a speaker for white noise (75 dB). Cocaine reinforcement was controlled by a lickometer circuit that monitored empty spout licking to operate a syringe pump (Model PHM-100VS, Med Associates, St. Albans, VT). A coupling assembly attached the syringe pump to the catheter assembly on the back of each rat and entered through a 5.0-cm diameter hole in the top of the chamber. The assembly consisted of a metal spring attached to a metal spacer with Tygon tubing inserted down the center, protecting passage of the tubing from rat interference. The tubing was attached to a counterbalanced swivel assembly (Instech, Plymouth Meeting, PA) that, in turn, was attached to the syringe pump. Events in the chamber and collection of data were controlled on-line with a Pentium computer that used programs written in the Medstate notation language (MED Associates).

2.4 Drug Preparation

Individual 20-ml syringes were prepared for each self-administration chamber prior to each daily session by diluting 4.0 ml of cocaine HCl stock solution (1.24 g cocaine HCl + 150 ml saline) with 16.0 ml of heparinized saline (0.1 ml 1000 IU heparin/60.0 ml saline) for a dose of 0.33 mg/infusion (Grigson and Twining, 2002; Puhl et al., 2009; Twining et al., 2009; Wheeler et al., 2008).

2.5 Data Collection

Habituation training, self-administration training, and extinction testing were conducted during the light phase of the light/dark cycle.

2.5.1 Habituation Procedure—Rats were habituated to the operant chambers for 1 h/day for four days prior to the beginning of self-administration training. During this time, each rat was maintained on a water restriction regimen in which they received 1-h daily access to water in the operant chamber via the right (“active”) spout, while the centrally located (“inactive”) spout was empty. Twenty-five ml of water was provided in the home cage overnight. Thereafter, the rats were returned to ad lib access to water for the duration of the study.

2.5.2 Phase I – Self-administration Training—Self-administration training began immediately following the 4-day habituation phase. Each rat was trained during daily 2 h sessions for 13 days. Specifically, rats were placed in the operant chambers in darkness. Immediately upon initiation of the 2 h session, the white noise was turned on, the right and

center empty spouts advanced into the chamber, and the cue light above the active spout was illuminated. A fixed ratio (FR) 10 schedule of reinforcement was implemented initially (Days 1–6). During this time, completion of 10 licks on the “active” spout was followed by a single intravenous (i.v.) infusion of 0.33 mg cocaine over six seconds. Drug delivery was signaled by offset of the stimulus light, spout retraction, and onset of the tone and houselight. The tone and houselight remained on for a 20-sec timeout period. Responding on the center “inactive” spout was without consequence throughout. During the final seven days of training (Days 7–13), the reinforcement schedule was increased to an FR20. All rats were returned to their home cages after completion of the 2 h session.

2.5.3 Phase II -Vehicle/RPEC Transplantation—Following self-administration training, rats were matched on the number of cocaine infusions taken on Trials 12 and 13 and divided into Low (n = 25) and High (n = 59) drug-takers. Low drug-takers averaged 16 or less infusions/2h, while High drug-takers averaged 17 or more infusions/2h. Low and High drug-takers were then tested for cocaine-seeking after 2 days of home cage abstinence (n = 12 Low; n = 21 High) or after 14 days of home cage abstinence (n = 13 Low; n = 38 High). Seven days into the 14-day abstinence period, rats in this condition received either transplantation of RPECs (20K cells/hemisphere) or empty beads (Vehicle) bilaterally into the medial shell of the NAc (Day 14 Vehicle Low: n = 7; Day 14 Vehicle High: n = 16; Day 14 RPEC Low: n = 6; Day 14 RPEC High: n = 22).

2.5.3.1 Transplantation into the NAc: RPECs were originally derived from fetal donor eyes via a confidential independent donor program (Advanced Biological Resources, Alameda, CA) and prepared into a cell line by passaging, expansion, sorting and frozen into individual aliquots of 2×10^6 cells (Titan Pharmaceuticals, Inc., Somerville, NJ). These cryovials of RPECs were provided to Dr. Subramanian for research via material transfer agreement. Prior to transplantation, these cells were thawed from -80°C storage and grown in tissue culture as we have previously described (Subramanian et al., 2002). On the day before transplantation, the monolayer was scraped and the cells were allowed to attach to pre-hydrated microcarriers as we have previously described (Subramanian et al., 2002). Attachment to microcarriers allows RPEC cells to assume their normal polarized biological status and promote graft survival. Under general anesthesia and adequate analgesia, rats were placed in a stereotax with the skull level and the tooth bar set at -5.0 . All NAc transplants were delivered at a rate of $1\mu\text{l}/\text{min}$ using a $10\mu\text{l}$ Hamilton syringe attached to a 27gauge needle, with an inner diameter of 0.21mm. Empty collagen beads or RPECs attached to beads (20K/hemisphere in $1\mu\text{l}$) were transplanted bilaterally at the following coordinates: AP = $+2.7$ from bregma, ML = ± 1.2 from the midline, DV = -6.8 from dura. Twenty thousand cells/hemisphere was chosen because 20,000 VTA neurons reportedly project into the NAc (Nair-Roberts et al., 2008). Following completion of the transplantation procedure, the surgical hemostasis was assured, the scalp was sutured in layers and the rats were allowed to recover from the anesthesia before they were returned to their home cages. Post operative analgesia (carprofen 5mg/kg; subcutaneously) was provided as needed under careful monitoring of board certified veterinary staff. No perioperative or postoperative immunosuppression was utilized.

2.5.4 Phase III – Extinction Testing—The drug-seeking test occurred either 2 or 14 days after the final drug self-administration trial. During this test, all rats were placed in the experimental chamber for a 2 h extinction session as described in self-administration training, with the exception that no drug was delivered. The number of infusion attempts, then, served as the dependent measure.

2.6 Histology and immunohistochemistry

Following the end of behavioral testing all rats were euthanized under deep general anesthesia. On average, animals were euthanized 8 to 12 weeks after their last cocaine self-administration test and a minimum of 8 weeks after RPEC or empty carrier transplantation in all animals. In all animals that were used for behavioral analysis, histological examination was performed with the exception of 6 brains from the first replication that were saved for future biochemical analysis and fresh frozen. Thus, all rats, save 6, were perfused with intracardiac cold buffered heparinized saline followed by 4% paraformaldehyde under deep general anesthesia when they were euthanized. The brain was rapidly dissected out and cryoprotected prior to coronal sectioning in series at 60µm thickness on a microtome. A whole series was stained with cresyl violet and evaluated (T.S.) to ensure accurate graft placement and overall health of the tissue in each brain in a blinded fashion (i.e., with no knowledge of the behavioral status of these animals). Immunostaining was performed using standard techniques that we have previously published using a rabbit polyclonal antibody for TH (Pelfreez Biologicals) and mouse monoclonal antibody for DDC (Abcam) – the 2 enzymes involved in the synthesis of dopamine. All antibodies were developed using standard ABC technique with DAB tertiary and was timed with appropriate controls. Design based stereology was performed for graft enumeration and for host VTA TH enumeration using techniques we have previously published (Gilmour et al., 2011a; Gilmour et al., 2011b; Lieu et al., 2012; Subramanian et al., 2002) for TH positive neurons. DDC staining was analyzed using NIH J for staining intensity.

3. RESULTS

As described, some experimental subjects were lost due to failed catheter patency. Others failed to survive the transplant surgery, or had poorly placed beads (for the vehicle controls) or RPEC grafts (see Histology section 3.3). The final sample sizes were: Day 2 Low: n=12; Day 2 High: n=21; Day 14 Vehicle Low: n=7; Day 14 Vehicle High: n=16; Day 14 RPEC Low: n=6; Day 14 RPEC High: n=22. All data were analyzed with Statistica7 (StatSoft, Tulsa, OK) using 2 × 3 analysis of variance (ANOVA) varying group (low vs. high) and treatment group (Day 2, Day 14 Vehicle, and Day 14 RPEC), independent group t-tests, and paired t-tests.

3.1 Terminal number of infusions of cocaine/2h

As shown in Figure 1, the high drug takers took more infusions of cocaine during the terminal trials (i.e., averaged across trials 12 and 13) than did low drug takers and this was true for rats that ultimately would serve in each of the three treatment conditions (Day 2, Day 14 Vehicle, and Day 14 RPEC).

This conclusion was supported by a significant main effect of Group, $F(1,78) = 191.04$, $p < .001$. Follow up t-tests confirmed that high drug takers took more infusions of cocaine than low drug takers for each of the three treatment conditions: Day 2: $t(31) = 9.09$, $p < .001$, Day 14 Veh: $t(21) = 8.59$, $p < .001$, and Day 14 RPEC: $t(26) = 7.68$, $p < .001$, respectively. There was no significant main effect for future Treatment Condition ($p < 0.05$) and no significant Group $\times$ future Treatment interaction ($p < 0.05$). Follow up t-tests also confirmed that high drug takers in the Day 14 RPEC treatment condition took fewer infusions of cocaine than high drug takers happened to take in the Day 2 treatment condition, $t(20) = 2.38$, $p = .027$, but the same number of infusions as the high drug takers in the Day 14 Vehicle treatment condition, $t(15) = 1.29$, $p = .129$.

3.2 Mean number of Infusion Attempts/2h

As shown in Figure 2, overall, the high drug takers attempted to take more infusions than did the low drug takers during extinction testing.

This conclusion was supported by a significant main effect of Group, $F(1, 78) = 16.66$, $p < .001$. Follow up t-tests revealed that, in fact, high drug takers in the Day 2 and Day 14 Vehicle treatment condition made more infusion attempts to take cocaine than did rats with a history of low drug taking $t(31) = 2.89$, $p = .001$, and $t(21) = 3.57$, $p < .001$, respectively. There was, however, no significant difference in drug-seeking between rats with a history of high and low drug taking in the Day 14 RPEC treatment condition, $t(26) = 0.92$, $p = .367$. Further, while there was a tendency for the number of infusion attempts to be greater in the RPEC treated low drug-takers, this trend did not attain statistical significance relative to either the Day 14 Vehicle treated low drug-takers, $t(5) = -2.05$, $p < 0.1$, or the low drug-taker Day 2 controls, $t(5) = -0.67$, $p < 0.5$. The main effect of Treatment group also was not significant, $p < 0.05$. Nor was the Group $\times$ Treatment interaction, $p < 0.05$. Bilateral transplant of RPECs into the NAc, then, served to rescue rats with a history of high cocaine self-administration from cue/context-induced reinstatement when tested following a 2 week abstinence period. Indeed, these RPEC transplanted subjects performed like low drug-takers.

3.3 Histology

Day 2 animals were not subject to detailed histology as they did not have grafts ($N=33$). However, they were used as controls for all of the histological analysis for immunohistochemistry. Histology was conducted on 45 out of 51 (88%) of the rats that received RPEC or Vehicle transplants. The remaining 6 brains (12%) were harvested without perfusion and fresh tissue homogenates prepared for future testing. Histological assessments were performed blinded to behavioral results. Any animal that had bilateral grafts in the NAc made it to the primary analysis. Rats that had either misplaced grafts or had no locatable grafts despite grafting were not used for analysis as their numbers were small and sub group analysis was impossible.

Transplants were located in the shell and/or the core of the NAc bilaterally in all transplanted subjects that contributed data to the final analysis. A representative coronal section stained with cresyl violet through the NAc is shown in Figure 3 to illustrate graft placement and the histological characteristics of RPECs attached to microcarriers

transplanted into the NAc. Rats that received only unilateral accurate grafts were excluded from the analysis. These subjects also did not exhibit behavioral benefits and they were too few in number to permit separate analysis as a sub-group.

Quantitative estimation of RPEC graft locations in each animal using unbiased design based stereology revealed each graft site to have 19,433+/- 421 RPEC cells. In this context, it is important to reiterate that these human origin cells were transplanted into outbred rats without any immunosuppression of the host.

3.4 Immunohistochemistry for Tyrosine hydroxylase (TH) and Dopa decarboxylase (DDC)

Examination of the graft sites showed the presence of TH positive RPECs attached to beads located within the NAc (Figure 4).

In contrast, the empty beads in vehicle treated animals located in the NAc had no TH positive profiles. Design based unbiased stereology of TH positive cell bodies in the host VTA was performed. Total estimates for TH positive neurons in the VTA of inexperienced rats (i.e., healthy normal adult rats, N=10) obtained from an archived normal histology tissue bank (T.S.) and processed exactly the same as was the tissue in this experiment were a mean of 18,098 cells/hemisphere. This stereological estimate is consistent with estimates in published literature. In comparison, cocaine experienced high drug taking rats that received the empty microcarrier vehicle transplant (Vehicle Only), showed a significant reduction (73%) in TH positive dopaminergic neurons in the VTA. Cocaine experienced high drug taking rats that received bilateral RPEC transplants showed better preservation of TH positive VTA neurons as estimated by stereology (40% reduction) – a finding that was statistically significant, Student t-test at $p < 0.035$, N=10 each, RPEC and vehicle treated animals (Figure 5).

Low drug takers did not show a statistically significant change in TH positive VTA cell estimates compared to controls, $p > 0.05$. These data suggest that there are benefits to RPEC graft placement into the NAc leading to improved survival of VTA dopaminergic neurons in high drug-taking rats.

DDC staining demonstrated no differences at the level of the VTA in any group. However, in the NAc there were differences in DDC immunostaining. Specifically, all cocaine experienced animals (i.e., subjects from all three treatment groups) had significantly higher expression of DDC in the NAc than the cocaine-naïve normal controls, $p < 0.0003$, N=10 each for Day 2 animals, RPEC, vehicle, and naïve normal controls. Compared to Day 2 animals that did not receive any transplants, both RPEC and vehicle treated animals tended to have increased expression of DDC in the NAc, particularly in the high drug taking animals, but this trend was not statistically significant (Figure 6).

Low drug taking animals had similar DDC expression to Day 2 (no transplant) animals (data not shown).

3. DISCUSSION

Our results demonstrate the usefulness of a cell grafting approach to mitigate drug reinstatement behavior in a rat model of cocaine seeking. This serves as proof of concept that RPEC grafts could potentially be useful in refractory drug addiction. As technology has improved, neurosurgical interventions like deep brain stimulation (DBS) have had clinical application and have become United States Food and Drug Administration (US FDA) approved standard therapy in neuropsychiatric disorders like medication refractory clinical depression, Tourette syndrome, obsessive compulsive disorder, dystonia, tremor and PD as the risk versus benefit ratio for invasive therapy has improved dramatically (Altinay et al., 2015; Guercio et al., 2015; Hamani et al., 2014; Kohl et al., 2014; Morishita et al., 2014). Neurosurgical interventions like DBS and GDNF replenishment into the NAc have been tried successfully in rat models of cocaine addiction (Airavaara et al., 2011; Girard et al., 2006; Green-Sadan et al., 2003; Green-Sadan et al., 2005; Lu et al., 2009; Pickens et al., 2011; Vassoler et al., 2013). Moreover, there is growing consensus that the neurobiological basis for drug addiction is becoming more clear and interventions that correct this pathophysiology may have tangible clinical benefits (Benarroch, 2015; Guercio et al., 2015; Pereira et al., 2015; Yadid et al., 2013).

As alluded to, previous attempts to use glial derived neurotrophic growth factor - GDNF (Airavaara et al., 2011; Green-Sadan et al., 2003; Green-Sadan et al., 2005; Lu et al., 2009) and DBS (Hamilton et al., 2015; Rouaud et al., 2010; Vassoler et al., 2008; Vassoler et al., 2013) to mitigate cocaine reinstatement have been reported. In contrast to these methods, the use of RPEC grafts has the advantage that these grafts secrete L-dopa, the precursor for dopamine, and a variety of growth factors including GDNF, PEDF and a large number of anti-inflammatory cytokines. Such a versatile graft that can be grown in tissue culture, well characterized for purity and screened for teratogenicity, has the potential to replenish neurotransmitter deficiencies in the NAc, while providing growth factors and immunomodulates to its immediate environment in the brain. Moreover, the use of RPECs allows us to forego chronic daily immunosuppression, as these xenografts survive for potentially the life of the host due to their ability to produce anti-inflammatory cytokines.

Our data support the notion that RPEC grafts into the NAc can provide better preservation of the VTA dopaminergic neurons that project to the NAc. We did not evaluate the dendritic morphology of the NAc or the dorsal striatal neurons in transplanted animals. In view of recent studies that show the critical role of synaptic and dendritic pathobiology (Villalba and Smith, 2013) seen in cocaine reinstatement studies (Shen et al., 2014), such studies may be of use in future transplantation experiments to evaluate whether VTA neuronal preservation will provide reversal of dendritic changes in post-synaptic NAc neurons. Our study also provides support to the growing consensus that the VTA-NAc pathway has a significant role in reinstatement of drug-seeking behavior and, as such, adds an additional line of evidence to recent related optogenetic studies in the VTA-NAc pathway (Adrover et al., 2014; Bocklisch et al., 2013; Chandra et al., 2013; McCutcheon et al., 2014; Stefanik et al., 2013).

The use of RPEC has numerous advantages. We demonstrated previously that fetal origin RPECs attached to microcarriers can be transplanted into the dopamine-depleted striatum for

the treatment of PD (Stover et al., 2005; Subramanian et al., 2002). After initial success in animal models we performed the phase 1 human trial (Stover et al., 2005; Stover and Watts, 2008; Subramanian and Deogaonkar, 2013). In this trial we were able to demonstrate safety and efficacy for such transplants in PD. In a phase 2 clinical trial, the commercial study sponsor of these studies switched to a post-natal source of RPECs that were again attached to microcarriers and transplanted into a larger cohort of patients. In this study, both placebo and the test groups demonstrated clinical benefit and there were no statistical differences between the placebo and test groups (Gross et al., 2011). One individual from this study that received post-natal RPEC transplantation who died from causes unrelated to the surgery has come up for autopsy examination. The published pathology from a single hemisphere from this subject showed poor survival of these post-natal RPECs (Farg et al., 2009) in this individual. However, several other transplantation studies in PD that used fetal RPEC attached to microcarriers have shown that such transplants are efficacious and without any side effects (Bu et al., 2014; Ming et al., 2009; Xue et al., 2013; Yin et al., 2012). Furthermore, a single fetal RPEC donation can be expanded in tissue culture such that over 50,000 patients could be potentially transplanted.

Current experiments show that bilateral transplants of RPECs into the NAc prevent reinstatement of drug-seeking behaviors after 2 weeks of abstinence, in high drug-taking rats. The most likely mechanistic explanation of the beneficial effects of NAc grafts on TH positive neurons in the VTA is via the retrograde transport of beneficial trophic factors secreted by the RPEC grafts and transported via the remaining intact VTA-NAc axons and their neuritic processes. Whether the observed behavioral benefits are mediated by the improved survival of dopaminergic neurons in the VTA-NAc pathway alone, or whether there is a contribution from the exogenous RPEC graft derived L-dopa secretion and/or local secretion of growth factors, cannot be distinguished in the present study. Another finding in these studies is the successful survival of RPECs in this xenograft paradigm without the need for any immunosuppression. This finding suggests that RPECs secrete immunomodulators that suppress the host immune response to these grafts in the brain (Reviewed in (Piquet et al., 2012)). Such secreted immunomodulators also may play a role in the observed behavioral benefits in these rats. We did not directly assess levels of secreted L-dopa, growth factors or cytokines at and around the graft sites or in the VTA. We also did not evaluate the fasciculus retroflex originating in the habenula that has been implicated as the source of neurodegeneration and cell loss in the VTA (Benarroch, 2015; Ellison, 1992; Pereira et al., 2015). These are drawbacks of this study. Future studies that evaluate RPEC graft secreted L-dopa, growth factors and cytokines *in vivo* and a more comprehensive evaluation of the neurobiological changes caused by RPEC grafts on the neuropathology induced by cocaine on the host brain are warranted.

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Highlights

- Some out bred Sprague-Dawley rats take more cocaine than others
- High drug-takers show greater reinstatement of cocaine seeking than Low drug-takers
- RPEC grafts into NAc rescue High drug-takers from reinstatement after 14d abstinence
- RPEC grafts into NAc preserve DA cell bodies in the VTA of High drug-taker rats
- RPEC grafts in NAc protect DA cells in VTA and rescue High takers from reinstatement

Figure 1. Mean ($\pm$ SEM) number of cocaine infusions/2 h averaged across terminal self-administration trials 12 and 13 for Low and High drug-takers as a function of later group assignment for extinction testing (Day 2, Day 14 Veh, Day 14 RPEC). * $p < 0.001$

Figure 2.

Mean number of cocaine infusion attempts/2 h for Low and High drug-takers for rats that received extinction testing after 2 (group Day 2) or 14 days of abstinence. Approximately half of the subjects in the Day 14 group received a bilateral empty bead vehicle transplant (group Day 14 Veh), and half a bilateral RPEC transplant (group Day 14 RPEC) into the medial shell of the NAc approximately 7 days prior to test. * $p < 0.001$

Figure 3.

A. Representative coronal section stained with cresyl violet through the NAc in a rat that received RPEC transplants showing accurate placement of the graft into the NAc shell. Note the needle tract and the entry wound in the cortex. B. High power view (20×) of graft site with the collagen beads. C. Details (60×) of the graft showing viable large brownish purple cells attached to the bead matrix (round profiles). The brown color is due to the melanin pigment in these RPECs. Animals were euthanized 8 to 12 weeks after their last cocaine self-administration test and a minimum of 8 weeks after RPEC or empty carrier transplantation in all animals

Figure 4.

A representative high power (40×) photomicrograph of TH immunohistochemistry from the NAc in a high drug taking RPEC grafted rat showing grafted microcarriers decorated with TH positive cells (arrows) with multiple TH positive RPECs. Animals were euthanized 8 to 12 weeks after their last cocaine self-administration test and a minimum of 8 weeks after RPEC or empty carrier transplantation in all animals.

Figure 5. Design based stereological estimates of TH positive neurons in the VTA in high drug taking animals that received vehicle (empty beads) versus RPECs attached to bead transplants bilaterally into the NAc. Error bars indicate coefficient of error (CE) and * $p < 0.035$

Figure 6. Densitometric measurements (NIH J) of DDC immunostaining bilaterally in the NAc of high drug taking rats transplanted with RPEC or vehicle compared to day 2 (non transplanted rats). These differences were not statistically different.