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Transplanted Human Neural Progenitor Cells Attenuate Motor Dysfunction and Lengthen Longevity in a Rat Model of Ataxia

Wesley M. Tierney[1], Toni L. Uhlendorf[1], Aaron J.J. Lemus[1], Bianca A. Ortega[1], Jesse Magaña[1], Jessica Ochoa[2], William Van Trigt[2], Angelica Cruz[2], Alex Kopyov[2], Oleg V. Kopyov[2], and Randy W. Cohen[1]

Abstract

The *spastic* Han Wistar (sHW) rat serves as a model for human ataxia presenting symptoms of motor deterioration, weight loss, shortened lifespan, and Purkinje neuron loss. Past studies revealed that human neural progenitor cells (NPCs) improved ataxic symptoms at 20 d posttransplantation in sHW rats. In this study, we investigated the fate and longer-term effectiveness of these transplanted NPCs. Rats were placed into four treatment groups: an untreated normal control group ($n = 10$), an untreated mutant rat control ($n = 10$), a mutant group that received an injection of dead NPCs ($n = 9$), and a mutant group that received live NPCs ($n = 10$). Bilateral cerebellar injections containing 500,000 of either live or dead NPCs were performed on mutant sHW rats at 40 d of age. Motor activity for all mutant rats started to decline in open field testing around day 35. However, at day 45, the live NPC-treated mutants exhibited significant improvements in open field activity. Similar improvements were observed during rotarod testing and weight gain through the completion of the experiments (100 d). Immunohistochemistry revealed few surviving human NPCs in the cerebella of 80- and 100-d-old NPC-treated mutants; while cresyl violet staining revealed that live NPC-treated mutants had significantly more surviving Purkinje neurons compared to mutants that were untreated or received dead NPCs. Direct stereotactic implantation of NPCs alleviated the symptoms of ataxia, acting as a neuroprotectant, supporting future clinical applications of these NPCs in the areas of ataxia as well as other neurodegenerative diseases.

Keywords

Purkinje cells, cerebellum, stem cell transplantation, *spastic* Han Wistar rat

Introduction

Ataxia is a human motor disorder where the main or known feature is the degeneration of Purkinje cells of the cerebellum[1]; as the disease progresses the symptomatic motor dysfunctions parallel neuronal loss[1,2]. Currently, negligible clinical alleviation of ataxic symptoms has been possible with a combination of physical therapy and drugs. Alternatively, the use of cell replacement therapy has shown notable promise to slow the progression of neuronal degeneration found in multiple neuronal disorders[3]. Cell replacement therapies for these disorders such as traumatic brain injury, Alzheimer's, Parkinson's, Huntington's, amyloid lateral sclerosis, and, of importance to our study, ataxia are currently being investigated[4,5]. Within treatments of ataxia, cerebellar Purkinje cells are usually the main target for cell replacement therapy[3,6].

Numerous in vivo experiments have been conducted on the efficacy of reducing neurodegeneration via direct Purkinje cell replacement in ataxic rodent models[3]. Researchers

[1] Department of Biology, California State University, Northridge, CA, USA
[2] Celavie Biosciences, LLC, Oxnard, CA, USA

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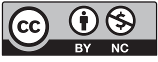
Corresponding Author:
Randy W. Cohen, Department of Biology, California State University, Northridge, CA 91330, USA.
Email: randy.cohen@csun.edu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found that injections of mesenchymal stem cells (MSCs) improved motor symptoms in the neurodegenerative Lurcher mice that exhibit progressive loss of Purkinje cells[7,8]. Murine MSCs were injected unilaterally into the cerebellum of newborn Lurcher mice[7]. After 1 mo, MSCs were found to have migrated into the white matter of the cerebellum, and the transplanted mutant mice showed significant improvements in rotarod performance compared to untreated Lurcher mutants. After 2 mo, a majority of MSCs did not survive; however, the transplanted cerebellar cells expressed neuroprotective growth factors such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, and glial cell-derived neurotrophic factor (GDNF), ultimately triggering increased survival of Purkinje cells[7]. In another experiment, neural stem cells (NSCs) harvested from postnatal day 1 wild-type mice were injected into the cerebella of 42 to 46-d-old C57BL/6 Machado–Joseph disease transgenic mice, alleviating motor dysfunction and reducing neuronal loss[9]. Histologically, these NSCs differentiated into both neural and glial cells with increased BDNF expression that correlated with improved Purkinje cell survival[9]. Researchers also utilized murine neural progenitor cells (NPCs) to facilitate recovery in spinocerebellar ataxia type 1 (SCA1) mice models[10]. Mouse NPCs were harvested and transplanted into 5-, 13-, and 24-wk-old SCA1 mice, and their activity scores were monitored over 8 wk posttransplantation. While no motor improvement occurred within 4 wk posttransplantation in the younger aged treatment groups, motor activity scores did show significant improvements in the 24-wk transplantation group, displaying healthier Purkinje cells and less degeneration. Yet no Purkinje cell replacements were detected despite the observation that NPCs had migrated into all cerebellar layers including the Purkinje cell layer. The researchers concluded that cell-to-cell contact was the probable mechanism for Purkinje cell rescue from these NPCs[10]. Finally, NSCs have also been used to slow Purkinje cell degeneration (PCD) in the *nervous* (*nr*) mouse model[11]. These *nr* mice show PCD beginning at 30 d of age characterized by abnormal and dysfunctional Purkinje cell mitochondria, behaviorally hyperactivity and progressive motor ataxia. The researchers transplanted NSCs into the cerebella of neonatal *nr* mice. At 180 d, the researchers observed increased synaptogenesis (via amplified dendritic growth), improved mitochondrial activity, enhanced motor function, and significantly less PCD[11].

Stem cell clinical trials have also been attempted to aid human ataxic patients. Patients with spinocerebellar ataxia type 3 have debilitating symptoms that affect their quality of life including unsteady gait[12] and poor balance and eye coordination, leading to overall motor dysfunction[13]. Human adipose tissue-derived MSCs were injected intravenously into ataxic patients at the concentration of 106 cells/kg of body weight[14]. Posttransplantation follow-up showed brief motor improvements with significant stabilization of their ataxic symptoms[14]. Obviously, more stem cell research is crucial to discover the perfect cell replacement technology that will improve the quality of life for ataxic patients.

Previous stem cell therapies in our laboratory have utilized the *spastic* Han Wistar (sHW) rat, a model for ataxia. This mutant rat has an autosomal-recessive gene mutation that results in the progressive loss of cerebellar Purkinje cells[15,16]. The onset of this disease can be identified in the sHW rat at 20 d of age by observing motor incoordination, hind leg rigidity, and fore limb tremors with Purkinje cell neurodegeneration visible at 30 d of age[15,16]. The sHW mutant Purkinje cells continue to degenerate up to day 60 to 65 when most animals die of respiratory complications and their inability to get food. In prior studies, it has been shown that Purkinje cell death in the cerebellum linked to glutamate excitotoxicity[16].

Our lab has examined multiple routes of various treatments to increase survivorship of Purkinje cells, improve motor ability, and augment lifespan in the sHW rat. For example, N-methyl-D-aspartate (NMDA) and non-NMDA receptor blockers significantly improved longevity in the sHW rat[17,18] while exercise[19] combined with the neurotrophic factor BDNF release decreased Purkinje cell death[20]. Although these various treatments significantly increased motor ability, reduced Purkinje cell loss, and enriched sHW mutant longevity, these improvements were not persistent as all treated mutants eventually succumbed before 80 d of age. To sustain the sHW rat's continued existence, there is a desire to find a more effective solution for neuronal survival.

Human NPCs obtained from Celavie BioSciences, LLC (Oxnard, CA, USA) have been previously tested as a potential cell therapy for a variety of treatments. These NPCs express the specific stem cell markers octamer-binding transcription factor 4 and sex-determining region Y-box 2. The multipotent characteristics of these NPCs suggest that this cell line may act as novel treatments for a variety of neurodegenerative diseases. In a recently completed Phase 1 trial, Celevie's NPCs were implanted into the putamen of seven patients with mild-to-moderate Parkinson's disease (PD). At the 4-yr follow-up study, six of the seven patients showed significant improvements in Unified Parkinson's Disease Rating Scale part III (measuring motor function) compared to their previous baseline scores[21]. While a definitive conclusion on NPC efficacy will require larger controlled PD trials, these initial results suggest that transplanted NPCs have a positive and lasting effect on neurodegenerative disorders.

Previous research in our lab examined various methods and dosage of Celavie's human NPC transplantation into 40-d-old sHW rat cerebella. First, carotid artery injection and unilateral direct cerebellar transplantations were compared in the 40-d-old sHW rat[22]. Researchers found that NPCs were present in the brain only after direct cerebellar injections rather than carotid artery infusion. In addition, significant amelioration of the sHW rat's ataxic symptoms was found only in those rats with direct injection of NPCs into the cerebellum (20 d posttransplantation)[22]. In our next

study, we examined whether increasing the NPC load with cerebellar bilateral injections could reduce symptoms and extend longevity in the sHW rat[23]. NPC-injected mutant rats showed significantly improved motor abilities and extended weight gain. After immunohistochemical staining of the brain tissue in these treated rats, the surviving human NPCs were observed to be migrating toward the damaged Purkinje cell layer in the sHW cerebellum and were shown to have calbindin-positive immunochemistry. The calbindin-positive NPCs may indicate early neuronal differentiation, but no cellular differentiation was detected since the experiment ended after 20 d posttransplantation (60 d of age)[23]. These data evoked an intriguing question: What would happen if we extended the experiment for another 40 d (with longer immunosuppression)?

Hence in our current study, we again transplanted Celavie's NPCs bilaterally into the cerebellum of the sHW rats. However, this experiment attempted to answer two relevant follow-up questions: (1) If we extend immunosuppression in sHW mutants treated with human NPCs, will the ataxic symptoms become completely reversed? (2) After lengthening treatment time, what do the transplanted NPCs differentiate into within sHW mutant cerebella?

Materials and Methods

Animals

sHW rats were obtained from California State University, Northridge's breeding colony. An equal mix of male and female rats were divided into four treatments: an untreated normal group ($n = 10$), an untreated mutant group ($n = 10$), a dead NPC-treated, mutant group ($n = 9$), and a live NPC-treated, mutant group ($n = 10$). Starting at 30 d of age, these animals were examined every 5 d for weight, rotarod, and activity scores. For histological analyses examining NPC longevity in sHW rats (sacrificing at 60 and 80 d of age), we used an additional set of untreated normal ($n = 6$) and live NPC-treated mutant rats ($n = 6$). Animals were housed in standard rat cages with access to Lab Diet 5001 rodent chow and water ad libitum. The room was maintained at a temperature of 22°C ± 1°C with a 12/12-h light/dark cycle. This study was approved by California State University, Northridge's (CSUN's) Institutional Animal Care and Use Committee IACUC (protocol #1718-002b).

Culturing Human NPCs

Human NPCs were obtained according to NIH Ethical Guidelines and have been fully described in previous studies[21–23]. Briefly, NPCs were grown for 13 doublings in ultra-low attachment culture flasks under feeder-free conditions in serum and xeno-free Eagle's essential medium (Hyclone, Logan, UT, USA), supplemented with Gem21 (Gemini Bio-Products, Sacramento, CA, USA), epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), basic fibroblast growth factor (Peprotech), transforming growth factor alpha (Peprotech), insulin-like growth factor I (Peprotech), leukemia inhibitory factor (Millipore, Temecula, CA, USA), calcium chloride (Fisher Scientific, Waltham MA, USA), Glutamax (Invitrogen, Carlsbad, CA, USA), non-essential amino acids (Hyclone), and an N2 supplement (Invitrogen), all of which were added at proprietary concentrations. The dead NPCs were implemented as a negative control for any paracrine effects from the live NPC inoculations. The dead NPCs were obtained from a live NPC population and then were placed without cryoprotectant into a −20°C freezer for 30 min to freeze kill the cells, and then stored in −80°C freezer until use. The suspended dead NPCs in culture medium were thawed for 1 h and then used immediately for transplantation in sHW mutants.

NPC Transplantation

Starting at 30 d of age, all rats were sedated using 2.5% isoflurane, and underwent subcutaneous implantation of an Alzet osmotic pump (Model 2ML4; 28-d duration; Durect Corp, Cupertino, CA, USA) to infuse cyclosporine (15 mg/kg/d) to suppress the rat's immune system. Ten days later, at 40 d, rats were anesthetized using chloral hydrate (350 mg/kg; Sigma, St. Louis, MO, USA) and received bilateral injections of 500,000 live NPCs or dead NPCs per section (1,000,000 cells total) into the cerebellum (AP −11.0 mm; ML ±2.0 mm; DV 5.5 mm). All experimental and control rats received chronic cyclosporine treatments via osmotic pumps which were changed every 23 d until the end of the experiment which was 60 d of age for dead NPC or untreated mutants or 100 d of age for live NPC mutants and untreated normal rats.

Motor Activity Testing

Starting at 30 d of age (just prior to osmotic pump implantation), all animals were tested for locomotion every 5 d for the duration of the experiment for motor activity using the MLK Activity Test System. The activity test consists of placing the rat in the center of the motor activity box (100 cm × 100 cm ABS black plastic open field box) and allowing them to move around freely for 2 min. Their movements were recorded using a web camera (Logitech Pro 9000, Logitech, Newark, CA, USA) coupled with Virtual Dub software that converted these data into a single motor activity score that summarized the distance traveled (centimeters) by each rat during their three, 2-min trials. Each rat rested 2 min between trials.

Rotarod Testing

To measure cerebellar decline, the rotarod, a horizontal rod that rotates at a constant speed of 16 RPM (Med Associates, Fairfax, VT, USA), was utilized to test balance, grip, and motor coordination (i.e., cerebellar function). Starting at 30 d of age (just prior to osmotic pump implantation), all rats

were tested on the rotarod every 5 d until they could no longer perform these exercises. Here, the rotarod was turned on and reached threshold speed after a few seconds. The rats were then placed on the rotarod, performing for three consecutive trials (each trial lasting for a maximum of 180 s), and the fall latency time (seconds) was recorded for each trial. Each trial was averaged as a single fall latency data point for each set of trials.

Tissue Processing

To prepare brain tissue for histological analysis, rats were transcardially perfused at various ages. For both the untreated and the dead NPC mutants, perfusion occurred at 60 d of age (20 d posttransplantation) while live NPC-treated mutants and untreated normal rats were perfused at day 60 of age (20 d posttransplantation), day 80 of age (40 d posttransplantation), or day 100 of age (60 d posttransplantation). All rats were perfused with a 0.9% saline solution followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS). The brains were harvested and post-fixed in PFA and kept until further processing. The brain tissue was kept in PFA for at least 2 d before being transferred to a 20% sucrose solution for at least 3 d to cryoprotect the brains before sectioning. Using a cryostat, the cerebellum was sectioned sagittally into 25-μm-thick sections and processed via immunohistochemical staining to identify surviving human NPCs or cresyl violet staining for Purkinje cell counts.

Immunohistochemistry Analyses

To help identify surviving human NPCs, the tissue was rinsed in a 1× wash buffer (Diagnostic Biosystems, Pleasanton, CA, USA) for 5 min. Endogenous peroxidase blocking as achieved by washing the sections in a solution consisting of 0.36% glucose (Fisher Scientific, Waltham, MA, USA), 0.01% glucose oxidase (MP Biomedicals, Solon, OH, USA), and 0.013% sodium azide (Fisher Scientific) in 1× PBS (Fisher Scientific) for 60 min, proceeded by a 5-min rinse in wash buffer. Permeabilization was achieved by rinsing the tissue in wash buffer consisting of 0.1% Triton X-100 (Fisher Scientific) for 30 min, followed by a separate rinse in washing buffer for 5 min. Next, the tissue was washed with serum blocking solution consisting of 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) for 20 min. Next, tissue samples underwent a 15-min rinse of avidin blocking solution (Vector Laboratories). Tissue samples were then washed for 15 min in a biotin blocking solution (Vector Laboratories), followed by another 1-min rinse in wash buffer. After that, samples were incubated with mouse anti-human nuclei MAB4383 (1:200; Millipore, Carlsbad, CA, USA) in Antibody Diluent (Diagnostic Biosystems) for 60 min. After primary antibody incubation, the tissue underwent a 5-min wash buffer rinse that was followed by a 45-min incubation with horse anti-mouse biotinylated secondary antibody (1:100; Vector Laboratories). Tissue was then rinsed for 5 min in wash buffer, incubated for 60 min in ABC kit solution (Vector Laboratories), and rinsed for another 5 min with wash buffer. The tissue was incubated in ImmPACT DAB Substrate (Vector Laboratories) for 5 min and then rinsed in wash buffer for another 5 min. Next, methyl green (Vector Laboratories) was used as a counter stain and the slides were incubated for 1 min. After stain incubation, the tissue was washed in deionized water (Thermo Scientific, Waltham, MA, USA) for 5 min, then dehydrated in a series of alcohols and xylene as follows: 95% ethanol (Decon Laboratories, King of Prussia, PA, USA), 100% ethanol (Arcos Organics, Waltham, MA, USA), and Xylene (Fisher Scientific). Finally, the tissue sections were cover-slipped with mounting medium (Poly Sciences, Warrington, PA, USA) and examined for the presence of human cells under an Olympus BX60 microscope with ToupView version 7.3 software.

Cresyl Violet Staining/Purkinje Cell Counting

To quantify Purkinje cell survival, cerebellar sections were stained with cresyl violet, a general nuclear stain of neurons. Slides were hydrated in a diluted series of ethanol solutions (100%, 95%, and 70%) for 2 min. For the final hydration step, the slides were placed in a dish containing distilled water for 1 min. Next, the slides were immersed in cresyl violet stain (Sigma Chemical) for 3 min, followed by a 2-min rinse in distilled water. The slides were destained in acetic formalin for 5 min and then rinsed in distilled water for 2 min. Next, the slides were dehydrated by 2-min immersions in 95% ethanol, then 100% ethanol followed by a second immersion in 100% ethanol. Finally, ethanol was removed with an immersion in xylene for 2 min. The slides were then covered with Corning cover slips (Fisher Scientific, Santa Clara, CA, USA), using Permount (Fisher Scientific) as a sealant.

To determine effectiveness of NPC treatment on Purkinje cell survival, we counted Purkinje cells along 350 μm transects within cresyl violet stained cerebellar sections from live NPC-treated mutant (100 d of age), dead NPC-treated mutant (60 d of age), untreated mutant (60 d of age), and untreated normal (100 d of age) groups. To determine the proximal and distal influence of these NPC transplants, we calculated the number of surviving Purkinje cells adjacent (<100 μm) to needle tract ("Proximal") and farther (>1 mm) from the transplant site ("Distal"). The recognizable needle tract was used as the focal point for both the live NPC and dead NPC treatment groups. In the untreated normal and mutant groups, we utilized a rat brain atlas and the coordinates of the injection site (AP −11.0, ML ±2.0, DV 5.5) to determine where the probable focal point would be located in each series of cerebellar slices. This stereologic method allowed us to account for the lack of needle tracts in the untreated groups as we used the same cerebellum sections to count surviving Purkinje cells. Two observers, who were blind to treatment, performed the cell counts using an Olympus microscope at 400× magnification. Each counter

randomly selected two separate Purkinje cell regions (per 350 μm transect) per slide, and their counts were then averaged per animal within the four experimental groups and evaluated for differences in survival.

Statistical Analysis

All values shown are means ± standard error of the mean. Repeated-measure analyses of variance (ANOVAs) were performed using SYSTAT analysis program to detect differences in weight, motor activity scores, and rotarod performance. A repeated-measures design was used to compare multiple measurements on the same individual animal in time, and these data are correlated to each other[24]. A one-way ANOVA was used to test differences in Purkinje cell counts among treatment groups. Tukey's pairwise post hoc analysis was then used to determine statistical differences between the means. Significance levels were set at P <0.05 for all tests.

Results

We examined the long-term effectiveness of human NPCs in the cerebella of the sHW rat, our model of ataxia. To determine efficacy of treatment, we examined weight gain, rotarod performance, open field scores, and Purkinje cell survival in four treatment groups: untreated normal HW ($n = 10$), untreated mutant sHW ($n = 10$), dead NPC-treated mutant sHW ($n = 9$), and live NPC-treated mutant sHW ($n = 10$). Only untreated normal HWs and live NPC-treated mutant sHWs were examined until 100 d of age. Dead NPC-treated mutant sHWs and untreated mutant sHWs were examined up until 60 d of age due to the average lifespan of the mutants without treatment and with treatment besides the NPCs being 60 d of age. Sacrifice of the rats at this age was mostly due to bioethical reasons of not prolonging/alleviation of suffering of the animals.

NPC's Effect on Weight Gain

To assess the health of the animals, weights were taken every 5 d until sacrificed (60 or 100 d of age depending on genotype and treatment). Statistical significance was found in weights (Fig. 1) across treatments during the experiment ($F = 3.04$, $P < 0.05$). Normal rats showed the usual progression of increased weight for the entire length of the experiment while live NPC-treated mutants displayed statistically similar results as the untreated normal groups ($P > 0.1$). In contrast, both untreated mutants and mutants transplanted with dead NPCs showed a weight plateau at 45 d of age, and then showed a slight decline in weight starting at day 60 (Fig. 1). Tukey's post hoc analysis disclosed that mutant rats that received live NPCs had significantly increased weight compared to dead NPC mutants and untreated mutants starting at 50 d of age ($P < 0.05$). Throughout the study, no significant difference was found between the untreated normal rats and the NPC-treated mutant rats after 60 d of age until 100 d of age.

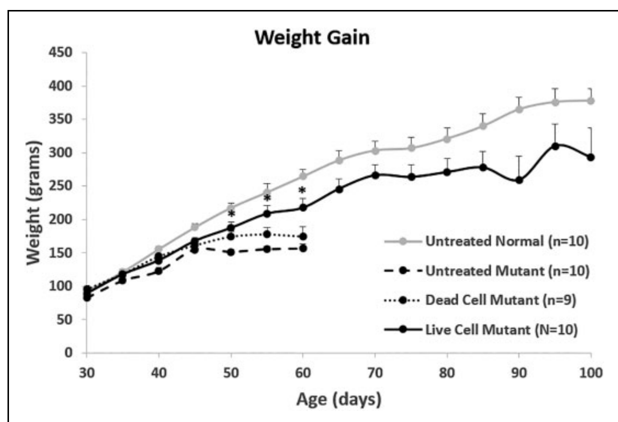

Fig. 1. Mean weight gain of the *spastic* Han Wistar rats was taken to assess the health of the live NPC-treated mutants ($n = 10$), dead NPC-treated mutants ($n = 9$), untreated mutants ($n = 10$), and untreated normal rats ($n = 10$) over the course of their lifespans. Statistical significance was found across treatments during the experiment ($F = 3.04$, $P < 0.05$). Specifically, the live NPC-treated rats showed statistically significant weight increases in comparison to both the dead NPC mutants and untreated mutants starting at day 50 ($P < 0.05$). Mutant rats that received live NPCs were not statistically different compared to untreated normal rats ($P > 0.05$). Data shown are mean ± standard error of the mean (repeated-measures analysis of variance; Tukey's post hoc test). Asterisk (*) indicates significant weight differences between live NPC and dead NPC mutants.
NPC: neural progenitor cell.

NPC's Effect on Rotarod Performance

The rotarod test was utilized to assess if the NPC treatment protects motor coordination as the cerebellar ataxia progressed in the sHW rats (Fig. 2). Every rat was tested for rotarod performance (fall latency times) starting at day 30 of age and continuing every 5 d until they were sacrificed (60 or 100 d of age). Statistical significance was found across treatments ($F = 25.03$, $P < 0.001$). Mutant rats that received the live NPC treatment showed statistically consistent fall latency times in comparison to dead NPC-treated mutants starting at day 35, which continued until the end of the experiment ($P < 0.05$). Additionally, untreated mutant rats displayed a statistically significant decrease in rotarod performance compared to mutants treated with live NPCs at day 45 lasting until the end of the experiment ($P < 0.05$). Tukey's post hoc analysis showed that live NPC mutants and normal untreated mutants had statistical similar fall latency scores up to day 75 ($P > 0.05$). However, at 80 d, live NPC-treated sHW rats exhibited reduced rotarod scores (compared to normal controls), which continued until the end of the experiment ($P < 0.05$).

NPC's Effect on Open Field Performance

As ataxia progresses, sHW mutants lose function of their hind legs. This progressive decrease in locomotor activity

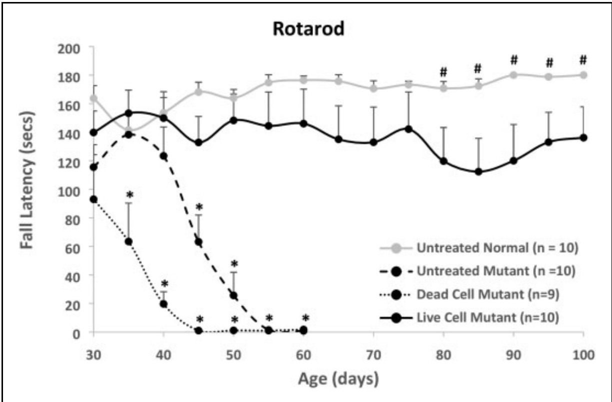

Fig. 2. Rotarod accessed motor coordination of the animals by measuring the mean fall latency (seconds) of live NPC-treated mutants ($n = 10$), dead NPC treated-mutants ($n = 9$), untreated mutants ($n = 10$), and untreated normal rats ($n = 10$) starting at 30 d of age. Statistical significance was found across treatments ($F = 25.03$, $P < 0.001$). Dead NPC-treated mutants showed significant differences compared to live NPC-treated rats ($P < 0.05$) starting at day 35. Untreated mutant fall latencies were also statistically decreased compared to the live cell mutants ($P < 0.05$), but these differences started later at day 45. Untreated normal rats and live NPC mutant rats had statistically similar rotarod scores up until day 75 ($P > 0.05$). However, starting at day 80, their fall latencies became statistically reduced ($P < 0.05$) for the rest of the experiment (100 d). Data shown are mean $\pm$ standard error of the mean (repeated-measures analysis of variance; Tukey's post hoc test). Asterisks (*) indicate significant differences in fall latency between live NPC and dead NPC mutants or untreated mutants; Pound symbols (#) indicate differences between live NPC mutants and untreated normal rats.
NPC: neural progenitor cell.

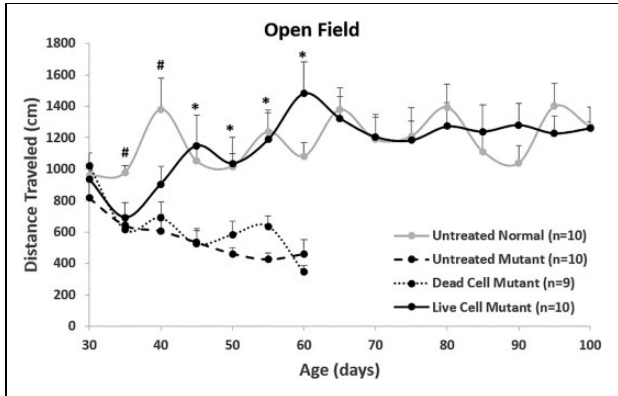

Fig. 3. Open field test was used to assess cerebellar performance in live NPC-treated mutants ($n = 10$), dead NPC-treated mutants ($n = 9$), untreated mutants ($n = 10$), or untreated normal rats ($n = 10$). Statistical significance was found across treatments ($F = 13.46$, $P < 0.01$). The live NPC-treated mutants showed a statistically significant drop ($P < 0.05$) in motor activity compared to normal controls early in the experiment (day 35). However, at day 45, there was a significant improvement in open field activities from live NPC mutants that were statistically similar to their untreated normal controls ($P > 0.05$). This trend continued until the end of the experiment. In contrast, dead NPC mutants and untreated mutants showed significant declines in motor activity by day 45 and were statistically different compared to live NPC mutants ($P < 0.05$). Data shown are mean $\pm$ standard error of the mean (repeated-measures analysis of variance; Tukey's post hoc test). Asterisks (*) indicate significant differences in fall latency between live NPC and dead NPC mutants or untreated mutants; Pound symbols (#) indicate differences between live NPC mutants and untreated normal rats.
NPC: neural progenitor cell.

can be detected via an open field test (Fig. 3). All rats were tested for open field activity starting at day 30 of age and every 5 d until they were sacrificed. Statistical significance was found across treatments ($F = 13.46$, $P < 0.01$). Starting at day 35, the live NPC-treated mutants had significantly decreased activity as seen in their open field scores compared to untreated normal rats ($P < 0.05$). This significant difference between live NPC-treated and untreated normal rats continued until day 45 when the live NPC mutants recovered with statistically similar activity test scores ($P > 0.05$). This rebound effect lasted throughout the entire experiment (100 d). In contrast to the live NPC mutants, the dead NPC-treated mutants and untreated mutants showed continuing motor decline until the rest of their lifespan ($P < 0.05$).

NPC's Fate at 60, 80, and 100 d of Age (20, 40, and 60 d Posttransplantation)

To examine survival of human NPCs implantation into the cerebellum of the live NPC-treated mutants at 60 ($n = 6$), 80 ($n = 6$), and 100 ($n = 10$) d of age (Fig. 4), immunohistochemical staining of tissue sections using human nuclear antibodies was performed, and subsequently examined for the presence of surviving human cells in the mutant rat cerebellum at various ages. Panel A: At 20 d posttransplantation (60 d of age), human cells are present near the needle track (arrows) and observed to be migrating toward the damaged Purkinje cell layer. Panel B: At 40 d posttransplantation (80 d of age), NPCs are still present (arrows), but in reduced presence. There were no longer any human NPCs that showed any migration from the needle track. Panel C: At 60 d posttransplantation (100 d of age), NPCs were very hard to find in situ. The few human cells that were present (arrows) were also misshapen, perhaps indicating a degenerative state. Clearly, the NPCs did not survive very long in the sHW rat cerebellum past 20 d despite chronic treatment with cyclosporine.

NPC's Effect on Purkinje Cell Survival

To determine the effect of NPC implantation on Purkinje cell survival, cerebellar slices from all treatment groups were mounted on slides and stained with cresyl violet and the surviving Purkinje cells were counted (Fig. 5). Two sets of

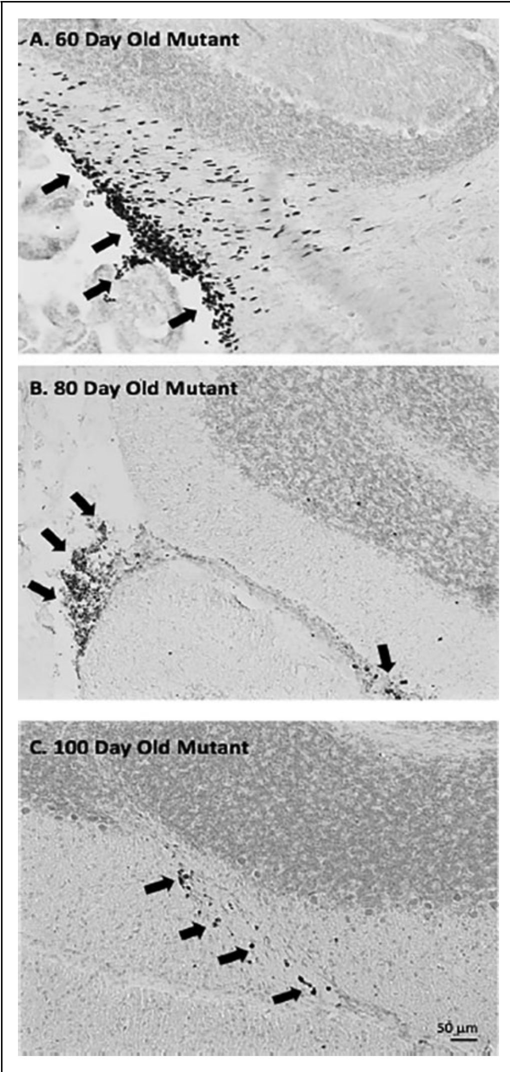

Fig. 4. Representative photomicrographs show the location of surviving human NPCs in the cerebellums of 60, 80, and 100-d-old mutant *spastic* Han Wistar rats (all transplanted at 40 d of age). The black arrows spotlight the location of the existing NPCs stained with anti-human antibodies. Image A (60-d-old/20 d posttransplantation) indicates the needle tract and the migration of NPCs toward the Purkinje cell layer. Image B (80-d-old/40 d posttransplantation) shows reduction of surviving NPCs in the cerebellum. Image C (100-d-old/60 d posttransplantation) indicates the location of very few surviving NPCs in the mutant cerebellum. Calibration bar in Panel C refers to all images.
NPC: neural progenitor cell.

cell counts were conducted: Proximal or adjacent (<100 µm) to the injection site or an anatomical similar area (for noninjected animals) and distal or 1 mm away from the injection site or anatomical similar areas. For the proximal cell counts (Fig. 5A), analysis showed that there were statistical differences across all treatments ($F = 12.39$, $P < 0.005$). Mutants that received live NPCs had statistically more surviving Purkinje cells per 350 µm transect compared to those mutants

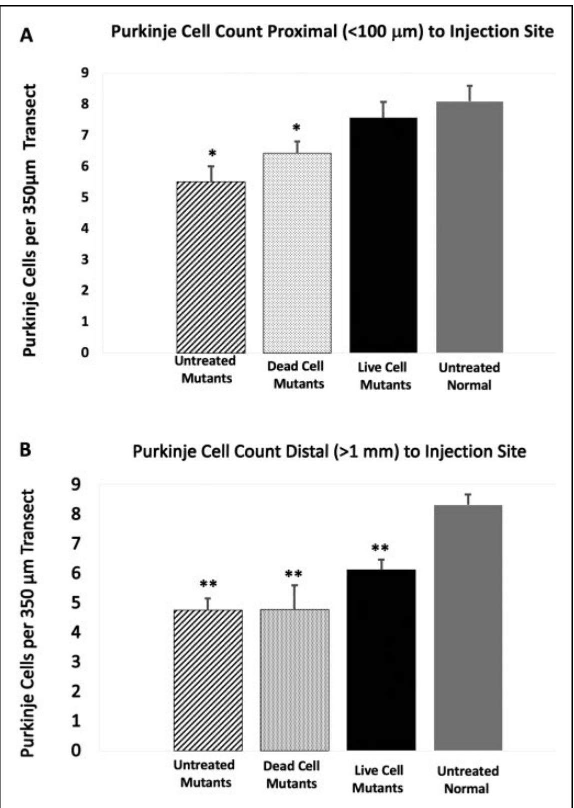

Fig. 5. To determine effectiveness of NPC treatment on Purkinje cell survival, we counted Purkinje cells along 350 µm transects within cresyl-violet-stained cerebellar sections from live NPC-treated mutant (100 d of age), dead NPC-treated mutant (60 d of age), untreated mutant (60 d of age), and untreated normal (100 d of age) groups. We calculated the number of surviving Purkinje cells within 100 µm from the needle tract (Proximal) and > 1 mm from the same track (Distal). Two observers, who were blind to treatment, performed the cell counts using an Olympus microscope at 400× magnification. Two randomly selected Purkinje cell regions (per 350 µm transect) were averaged per animal and evaluated for differences in survival. Proximal to the injection site (A), the results showed that the live NPC treat mutants showed a statistically similar number of Purkinje cells compared to the normal untreated rats ($P > 0.05$). In contrast, live NPC-treated mutants had significantly greater numbers of surviving Purkinje cells as compared to dead NPC mutants ($P < 0.05$) and mutant untreated rats ($P < 0.001$). These differences disappeared when Purkinje cell counts were accomplished farther away from the transplant site ("Distal"). Figure 5B showed similar reduced Purkinje cell numbers among all mutant groups regardless of treatment ($P > 0.05$) and these reduced cell counts were statistically lower than the normal controls ($P < 0.05$). Data shown are mean ± standard error of the mean (analysis of variance; Tukey's post hoc test). Single asterisk (*) indicates significant differences in Purkinje cells counts between live NPC mutants and dead NPC mutants; double asterisks (**) indicate significant difference in Purkinje cells survival between mutants and untreated normal rats.
NPC: neural progenitor cell.

that received dead NPCs ($P < 0.05$) and untreated mutants ($P < 0.05$). Mutants that received live NPCs had statistically similar amounts of Purkinje cells to that of normal untreated

rats ($P > 0.05$). Figure 5B shows cell counts distal to the injection site. Analysis showed that there were statistical differences among the treatments ($F = 13.39$, $P < 0.001$). Untreated normal rats had statistically higher amounts of Purkinje counts as compared to all other groups. However, unlike the proximal count data, no statistical differences were found among live NPC-treated mutants and mutant untreated or dead NPC-treated mutants ($P > 0.1$).

Discussion

Previously, our lab observed that Celavie's human NPCs survived, commenced migration, and stained positive for calbindin (an adult Purkinje cell marker) at 60 d of age (20 d posttransplantation) in the cerebella of our ataxic rat model, the sHW rat[23]. Additionally, these ataxic rats significantly improved their motor function and weight gain, indicating alleviation from the ataxic symptoms in this previous experiment. Given these earlier results, we hypothesized that if the transplanted NPCs were sustained in vivo longer (up to 40 more days), we would continue to observe improved motor function, weight gain, Purkinje cell survival, and, ultimately, detect maturation of the human NPCs into either mature glial or neuronal cells. In other words, given our previous results[23], these implanted NPCs should be an effective treatment for these ataxic rats.

At 100 d of age (60 d post-injection with human NPCs), the sHW rats showed remarkable improvements in weight and motor activity assays (behaving statistically similar to normal rats), suggesting that these cells indeed acted as an effective treatment for these ataxic mutants. It is indisputable that treatment by direct implantation of NPCs significantly altered the mutant sHW phenotype to resemble normal untreated rats in terms of weight gain (Fig. 1), rotarod performance (Fig. 2), and open field movement (Fig. 3). In contrast, untreated and dead NPC-treated mutant controls showed significant decreases in weight gain and impairment in motor function when compared to the live NPC-treated mutants. These behavioral abnormalities have been observed in all of our previous studies with untreated sHW mutants[15,16,22,23], suggesting that even transplanting dead NPCs into the degenerating cerebellum had no evident paracrine effects and in fact may have been detrimental to the ataxic rat. In addition, the Purkinje cell count experiment (Fig. 5) determined that the implanted NPCs had some unknown neuroprotective effects on in situ Purkinje cells. Mutants that received NPCs had significantly higher Purkinje cell counts as compared to mutants that received dead NPCs and untreated mutants adjacent to the injection site (Fig. 5A). Remarkably, mutants that were transplanted with NPCs had statistically similar numbers of Purkinje cells compared to normal untreated rats. In contrast, when analogous Purkinje cell counts were conducted farther from their inoculation site (>1 mm), the rescuing of Purkinje cells was not evident (Fig. 5B).

Despite the fact that the live NPC-treated rats survived well beyond their dead NPC counterparts, only few remaining NPCs were observed bordering the needle track (Fig. 4C) after 60 d of incubation (100 d old). As seen in Fig. 4A, implanted live NPC-treated mutants survived in great numbers 20 d posttransplantation. While no apparent differentiation or replacement was detected at 20 d posttransplantation, the surviving NPCs were observed to be migrating toward the damaged Purkinje layer, corroborating our earlier study[23]. In earlier studies in our lab Nuryyev et al. (2017) also showed that the NPCs stained positive for calbindin, an early marker for mature neurons (as well as a Purkinje cell marker as well), suggesting that the NPCs might have begun developing into Purkinje cells[23]. However, the 40- and 60-d posttransplantation immunostaining studies (Figs. 4B, C) showed very few surviving NPCs near the original needle track, indicating progressive cell death since their transplantation at 40 d of age. Our original hypothesis of alleviating the ataxic symptoms via cell replacement by human NPCs was not supported by these results, suggesting an alternative explanation of how these NPCs may have improved motor behavior and Purkinje cell survival over the 60 d posttransplantation.

So, why did these transplanted cells die? We pose two hypotheses to account for the lack of survival of human NPCs in the sHW rat. First, the xenogeneic transplantation grafting failure may simply be due to the response of foreign cells to the new host animal's milieu. Transplanted human cells have been shown to have poor, long-term survival in a xenobiotic environment like immunosuppressed rat brains[25–27]. Human NPCs may require the appropriate human-like environment like cell culture media that contain highly manipulated nutrients and growth factors providing the *in vitro* cells the appropriate environment to grow and differentiate. This novel environment is greatly altered in vivo and thus may be missing essential human growth factors in the rat brain, making long-term establishment of human neurons improbable depending on the cell type.

Other researchers have found similar long-term problems with human cell transplants into rodent brains. Erceg et al. (2010) found that human embryonic stem cells could be pushed to differentiate *in vitro* into mature Purkinje cells by a combination of growth factors and co-culturing them with mature mice cerebellum cells[28]. However, when these cells were transplanted into immunodeficient mice, the researchers discovered that most of the transplanted cells died without differentiating[28]. In another study, induced stroke rat models were implanted with human embryonic stem cells[29]. The researchers observed improvements in sensorimotor function, but discovered that less than 2% of the transplanted cells survived[29]. Another study noted the difficult survival of human transplanted cells in any host animal, including rats, and hypothesized that this was due to the lack of structural support and decided to test this by injecting the cells with human collagen[29]. These researchers injected human placental stem cells into the frontal cortex of induced

traumatic brain injury rat models with and without collagen. The human stem cells that were injected without collagen did not survive after 2 wk posttransplantation[30]. They theorized that without collagen to support them these transplanted human cells died because of an inability to engraft in situ[30]. Finally, stroke rat models were given intracranial injections of human NSCs either 2 or 24 h post-stroke. The rats were then split up into two treatment groups that were tested for cell survival after 3 or 35 d. Rats that had the transplanted human stem cells for 3 d had less than 1% of transplanted cells near the area of the injection; no human cells were found in the rat brains at 35 d[31]. It should be noted that long-term survival of human NPCs transplanted into rats has been achieved in a previous study[32]. This study looked at a rat model of PD and the effectiveness of transplanting human NPCs on amelioration of symptoms and survival. While survival was low, the existence of these transplanted cells within the Parkinson's rat was observed approximately 140 d posttransplantation[31].

A second but less likely concern was the possible failure of long-term immunosuppression with cyclosporine. While Nuryyev et al. (2017) successfully used cyclosporine and showed numerous NPCs in the sHW mutant cerebellum after 20 d in situ[23], it is possible that after long-term chronic usage (60 d posttransplantation), the cyclosporine failed to suppress the immune system entirely. We chose subcutaneous implantation of Alzet osmotic pumps to avoid adverse effects of daily injection in the rats and to ensure a chronic dosing of cyclosporine. This method has been used successfully by our lab[23] and others[25]. The cyclosporine could have become ineffective over time or our dosage of 15 mg/kg/d may not have been fully immunosuppressive as some researchers have shown that 12 mg/kg/d may not be fully immunosuppressive over time[25]. Cyclosporine has also been found to have limited effects on rodent immune systems[26]. A review of transplanted stem cell survival showed that cell survival was much lower in immunosuppressed organisms as opposed to organisms that were fully immunodeficient[27].

The number of factors playing into long-term transplantation and survival of human cells in rodent neural disease models includes cell type, transplantation region, method of transplantation, and immunosuppression/immunodeficiency of the host organism[31,33–35]. Remarkably, there is evidence of human NPCs surviving without any immunosuppression within rat brains[27,35]. Researchers found that after 4 to 6 wk posttransplantation, human neural NPCs survived in vivo without the use of cyclosporine or other immunosuppressants in a normal rat model[27]. Both immunosuppressed (with cyclosporine) and immunocompetent rats had human cells transplanted within their brains. While the immunosuppressed rats saw significantly higher levels of transplanted progenitor cells, there were still surviving human cells within the immunocompetent rats. Additionally, implanted human cells in both groups showed signs of differentiation into microglia. Thus, survival of human NSCs within rats without immunosuppression may be possible despite the xenobiotic nature[27]. Regardless, our use of immunosuppression in immunocompetent rats was performed to ensure that the transplanted xenobiotic cells lived long enough (60 d). In summary, we think that our use of immunosuppression (and the use of osmotic pumps to deliver chronically cyclosporine) was an aid to the transplanted NPC survival and not a deterrent. Thus, it is probable that human NPCs used are simply incapable of in vivo survival over long periods of time in this rat model.

While regenerative medicine has seen many positive results when it comes to stem cell therapy in human neurodegenerative diseases and their rodent models[21,23], full cell replacement does not always occur. However, regardless of the lack of replacement, differentiation, or survival of transplanted cells, our study showed that these NPC transplantations reversed ataxic symptoms over time. Fascinatingly, we have conducted an even longer pilot study by examining two NPC-treated sHW mutants (one male and one female) that were not sacrificed after the last cyclosporine pump was removed at 100 d of age. Both mutants survived for another year, suggesting that whatever paracrine effects the NPCs had within the mutant cerebella, the positive results lasted much longer even with no systemic cyclosporine present.

So, how could the dying NPCs continually protect the Purkinje cells in the sHW rat in the absence of direct Purkinje cell replacement? The Lurcher mouse strain presents symptoms similar to that of our ataxic rats[8], and stem cell therapy has been observed to alleviate their motor symptoms in a similar manner to our results[7]. When implanted with murine MSCs, Lurcher mice improved in overall motor scores[8]. However, like our results, the researchers found few surviving murine MSC after 2 mo. One of the possible hypotheses for this recovery was that the researchers found significant increases in various neurotrophic factors such as BDNF. Interestingly, not all labs that inject NPCs into ataxic models exhibited increased neurotrophic factors. An SCA1 mouse model that received murine NPCs did not observe any changes in neurotrophic factors[31]. These SCA1 mice received three stereotactic injections of NPCs into the cerebellum. The mice were tested for grip strength and on a rotarod for 8 wk. Only mice that received the progenitor cells at 24 wk old showed motor recovery; however, quantitative reverse transcriptase polymerase chain reaction analyses showed no significant increases in the neurotrophic factors BDNF, GDNF, or nerve growth factor. The treated SCA1 mouse Purkinje cells did retain some electrical functionality and maintained healthy morphology, but the researchers did not detect the biochemical mechanisms regarding how the injected cells safeguarded the Purkinje cells. The researchers did hypothesize that neuroprotection may have been due to cell-to-cell contact between the implanted progenitor cells and the host cells[36]. While not examined in our study, cell-to-cell contact may help explain the survival of the sHW rat Purkinje cells perhaps by reducing glutamate excitotoxicity. This protection from glutamate excitotoxicity via implantation of NSCs has been supported

by other researchers[37,38]. Finally, although we did not measure neurotrophic levels in mutant rat cerebella in this study, the cell count data (Fig. 5) do suggest that some permeable substance elicited long-term Purkinje cell protection. Further biochemical analysis would help confirm that these localized changes were induced by these neurotrophic factors.

Furthermore, the NPCs have not been the only cell type used for transplantation studies with ataxia models. Extensive work has been done on the PCD mouse model[39,40]. The PCD mouse shows similar motor dysfunctions and various cell death, including that of Purkinje cells. Bone marrow cell transplants, from both health and mutant mice, were performed to examine the effect the transplanted cells had on the PCD symptoms. Motor scores improved significantly after transplantation of the healthy bone marrow cells[39]. Interestingly, the nervous system damages found in the PCD model were not ameliorated. However, skeletal muscle structure, which is found damaged within the PCD, was ameliorated. The researchers believe that this and not any changes within the central nervous system were the cause of the improvements in the PCD model[39]. Later work by the same group has found that daily treatment with health bone marrow cells starting before neurodegeneration in the PCD mice was successful in ameliorating the neural damage found in PCD mice[40]. These results show the difficulty of cell transplantation using any type of cell line, but also the effectiveness of non-neural cell-based treatments for treatment of neurodegeneration. Further work with our sHW mutant rats could benefit from use of a non-neural, but established cell line seen effective in other ataxia models.

Conclusion

Our lab has established that Celavie Biosciences's human NPCs have been shown to be quite efficient at alleviating many of the symptoms of the sHW rat, our ataxic rat model. Our current hypothesis of Purkinje cell replacement stemmed from previous results in our laboratory, showing these progenitor cells surviving in large numbers and migrating 20 d posttransplantation[23]. However, in this study, the transplanted NPCs did not show complete differentiation to Purkinje cells nor did these xenotransplants survive much beyond 20 d posttransplantation. Despite a gradual reduction in their survival over time, the NPCs appear to have effected a lasting clinical recovery (improved weights, increased activity) and had significant neuroprotective outcomes on the mutant Purkinje cell population. Further experimentation would benefit from additional analyses of increased neurotrophic factors and perhaps examining cell-to-cell contact between the NPCs and Purkinje neurons as possible biochemical mechanisms preventing disease progression.

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Ethical Approval

This study was approved by Celavie Bioscience's Institutional Review Board.

Statement of Human and Animal Rights

This study was also approved by California State University, Northridge's Institutional Animal Care and Use Committee (Approval Number 1718-002b).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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ORCID iDs

Alex Kopyov https://orcid.org/0000-0003-0420-138X
Randy W. Cohen https://orcid.org/0000-0002-3945-7169

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