Human undifferentiated neural progenitor stem cells (NPC) alleviate motor dysfunction, repair morphological damage and extend life span in a spastic Han-Wistar model of cerebellar

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Introduction

Ataxia is a disease which leads to motor gait dysfunction and neural cell death. Specifically, Purkinje neurons of the cerebellum degenerate (Sawada et al., 2009) as the disease progresses, and fine motor coordination is lost (Jayadev and Bryd, 2013). Ataxia is both sporadic and familial with the sporadic being caused by gluten allergies and excessive alcohol consumption (Jayadev and Bryd, 2013). Genetic cerebellar ataxias come in many different forms, but most prevalently in Spinocerebellar Ataxia (SCA) 1-6. SCA 1-6 are caused by CAG repeats within different affected proteins, disrupting multiple cellular mechanisms especially within Purkinje cells (Orr, 2012).

As of now there is no effective treatment for any type of spino-cerebellar ataxia. However, the last few years have show that the use of replacement neurons (like stem cells) have potential not only to slow the progression of the disease, but also to augment and restore affected neurons, improving the patient's life. Cell replacement treatments for neurodegenerative diseases from Traumatic Brain Injuries, Alzheimer's Diseases, Ataxia, Huntington's, and Amyloid Lateral Sclerosis have been proposed as a treatment option (Lindvall and Kokaia, 2010; Tanna and Sachan, 2014). Specifically within Ataxia, replacing dying Purkinje neurons are generally the target for viable cell regenerative therapy (Orr, 2012; Nanri et al., 2011).

The *spastic* Han-Wistar (sHW) mutant rat that has an autosomal, recessive disorder, resulting in the progressive neurodegeneration of cerebellar Purkinje cells. Due to this mutation, this rat exhibits symptoms matching that of human ataxic patients such as motor incoordination, hind leg rigidity, and fore limb tremors. The sHW rats display shortened life spans of about 65 days (Cepeda et al., 2002) with cell degeneration beginning by postnatal day 30. Our lab's research has shown that the injection of neural progenitor cells (NPCs) from Celavie Biosciences was successful in increasing the longevity of the sHW rats. Our lab has looked at various methods of NPC transplantation such as carotid artery injection and direct injection into the cerebellum (Uhlendorf et al., 2016). They found that NPCs were present in the brain only after direct injections rather than carotid artery infusion. Nuryvev et al., (2017) recently examined NPC-injected mutant rats which showed improved motor abilities. After immunostaining of the brain tissue after three weeks post-transplantation, they discovered that surviving NPCs had started to migrate towards the damaged areas in the cerebellum and had shown calbindin-positive immunochemistry. Yet, no cellular differentiation was detected, asking the follow-up question: If given more time within the cerebellum, what would these human NPCs develop into?

This study addresses three questions: do transplanted NPCs continue to alleviate the ataxic symptoms over longer period of time; what do transplant NPCs differentiate into within this rat model of ataxia? And what are putative mechanisms the NPC's action? For this study, we implanted a total of 1 million NPCs (500,000 bilaterally) into the sHW cerebellum. As follow-up to our lab's previous work, we extended the time that the NPCs matured within the sHW cerebellum. Here we examine the effects of chronic NPC treatment via activity assays and histological analyses.

Methods and Materials

Animals: *Spastic* Han-Wistar rats (sHW; n=39) were obtained from California State University, Northridge's breeding colony. Rats (equal mix of males and females) were divided into four treatments: a non-mutant/normal group, a non-treatment mutant group, a dead NPC mutant group, and a live NPC group. Animals were housed in standard rat cages with access to Lab Diet 5001 rodent chow and water *ad libitum.* The vivarium room was maintained at a temperature of 22°C ± 1°C, with a 12/12-hour light/dark cycle. This study was conducted in an IACUC-approved, animal facility.

Culturing Human NPCs: Human NPCs were grown in culture medium consisting of ADCF MEM/EBSS basal medium, supplemented with EGF (epidermal growth factor; Peprotech), bFGF (basic fibroblast growth factor), IGF (insulin-like growth factor), TGFa (transforming growth factor alpha) LIF (leukemia inhibiting factor; Millipore), N2 (Invitrogen), and Gem 21 (Gemini Bioscience). Proteomic analysis of NPCs was performed by National Research Council of Canada.

NPC Treatment: Starting at 30 days of age, sHW rats were sedated using 2.5% isoflurane, and underwent subcutaneous implantation of an Alzet osmotic pump (Model 2004; 28 day duration) to infuse chronically cyclosporine (15 mg/kg/day) used to suppress the rat's immune system**.** Ten days later, at 40 days, mutant rats were anesthetized using chloral hydrate (350 mg/kg; Sigma), and received bilateral injections of 500,000 NPCs (1,000,000 cells total) or dead NPCs into the cerebellum (AP -11.0 mm; ML ±2.0 mm; DV 5.5 mm). Dead NPCs were used to control for any paracrine effects of NPC inoculations and were obtained from those live populations and frozen at -20°C until use.

Motor Activity Testing: Starting at 30 days of age (prior to pump implantation), all animals were tested every five days 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 x 100 cm ABS black plastic open field box) and allowing them to move around freely. Their movements were recorded using a web camera (Logitech Pro 9000) coupled with Virtual Dub software that converts these data into a single motor activity score that summarizes the distance traveled (centimeters) by each rat during the three, 2-minute trials.

Rotarod testing: To measure cerebellar decline, we used the rotarod to test balance, grip strength and motor coordination. The rotarod is a horizontally placed rod that rotates at a constant speed of 16 RPM. Starting at 30 days of age, the rats were tested every five days on the rotarod. The rat were placed on the rod while stationary, and then the rod was activated to start spinning. Each rat had three trials each lasting for a maximum of 180 seconds. If the animal fell off prior to the 180 seconds ending, the fall latency time (seconds) was recorded. The rats were tested until they could no longer perform the rotarod testing.

Tissue Processing: To prepare tissue for histological analysis, a select group of animals, just prior to death, were deeply anesthetized with chloral hydrate (400mg/kg) and transcardially perfused with 0.9% perfusion saline solution followed by 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS). The brains were harvested, and post-fixed in paraformaldehyde for at least two days before being transferred to a 20% sucrose/4% paraformaldehyde solution for 24 hours to cryoprotect the brains prior to sectioning. Using a cryostat, brain slices (20 µm thickness) were obtained from the cerebellum (parasagittal) and processed via immunohistochemical staining to identify surviving NPCs. Tissue was rinsed three times for 5 minutes each in washing buffer solution, and then incubated with human nuclei antibody for 1 hour at room temperature. Upon completion of incubation and application of second antibody and DAB, the tissue was incubated with the background stain Hoechst 33342 (1:10000; Invitrogen) for 5 minutes. The sections were then dehydrated in alcohols and cover-slipped with mounting medium contained 1X PBS, glycerol and n-propyl gallate (MP Biomedicals). Tissue sections from all animals were mounted on glass slides and examined for labeled hNPCs using Olympus BX60 fluorescent microscope with ToupView 7.3 software. Another set of cerebellar slices were utilized for Purkinje cell counts in each treatment. Slices were stained with the nuclear stain, Cresyl Violet, and Purkinje cells were counted by two observers who were blind to treatment. Counts occurred in two different stereologic regions of the cerebellum: proximal to transplant site; distal (> 1mm) from transplant site.

Results:

Mechanisms of Action: Paracrine Effect of OK99

Results in Increase in Number of Host's Purkinje Cells

Proteomic Analysis of the proteins secreted in the extracellular vesicles by OK99 Cells

- **Neuromodulin** - promotes neuronal growth, plasticity protection
- **14-3-3 Epsilon Protein** – reduces neurotoxicity induced cell death.
- **Insulin Like Growth Factor II** - Neuroprotective effect in aging brain
- **Activity Dependent Neuroprotective Protein** - Down-regulation contribute to dopaminergic neurodegeneration in PD
- **Ceruloplasmin** - Injections were shown to reverse cell death in substantia nigra (PD).

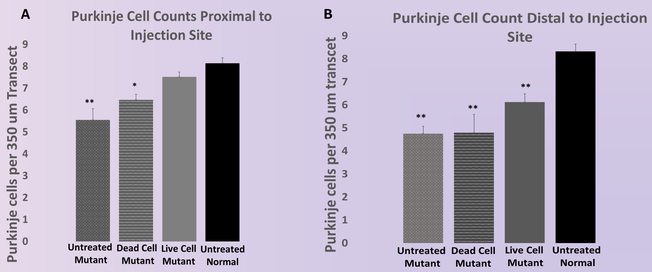

Figure 4. Purkinje cell counts of each group. **(A)** Cell counts proximal to the injection site or anatomically similar areas in rats that received no injections. Live NPC-treated mutants (n=10), dead NPC-treated mutants (n=9), mutant untreated rats (n=10) and untreated normal rats (n=10) were all counted. Analysis showed that there were statistical differences across all treatments (F=12.39, p<0.05). The live NPC-treated mutants showed a statistically significant larger amount of Purkinje cells as compared to dead NPC mutants (p<0.05) and mutant untreated rats (p<0.001). However, the live NPC treat mutants showed a statistically similar number of Purkinje cell compared to the normal untreated rats (p>0.05). **(B)** Cell counts distal to the injection site (>1 mm) or anatomically similar areas in rats that received no injections. Live NPC-treated mutants (n=8), dead NPC-treated mutants (n=6), mutant untreated rats (n=6) and untreated normal rats (n=6) were all counted. Analysis showed that there was statistical difference across all treatments (F=13.394, p<0.001. Post-hoc tests show that untreated normal rats has statistically higher amounts of Purkinje counts as compared to all other groups. No statistical differences were found between live NPC-treated mutants and mutant untreated or dead NPC-treated mutants. Data shown are mean±SEM, one asterisk indicating significant differences in Purkinje cells between live NPC mutants and dead NPC mutants; double asterisks indicating significant difference in Purkinje cells between live NPC mutants and untreated mutant rats (ANOVA; Tukey's Post-Hoc Test).

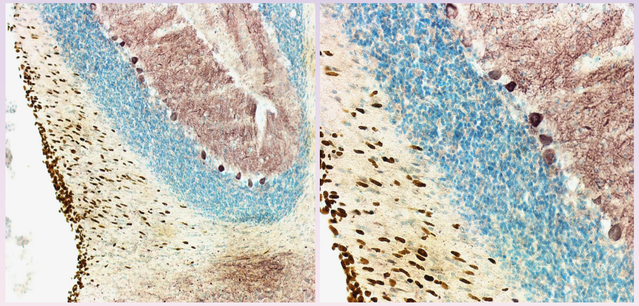

Figure 2. Immunohistochemical staining of the cerebellum of a mutant sHW rat that received injection of live human stem cells. Both images show human nuclei-labeled stem cells migrating from the needle tract towards the granule cell layer. Note the double-labeling of some human stem cells with calbindin. Human Nuclei (brown) at 1:200 dilution. Calbindin (purple) at 1:200 dilution. Methyl Green counterstain (blue). 100X and 200X, respectively.

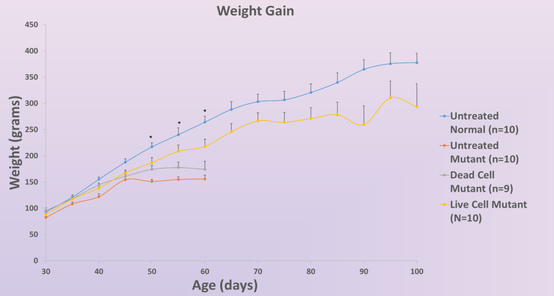

Figure 1. Weight gain of live NPC-treated mutants (n=10), dead NPC-treated mutants (n=9), untreated mutants (n=10) and untreated normal rats (n=10) from 30 days of age until age of sacrifice. Statistical significance was found across treatments during the experiment (F = 3.04, p < 0.05). The live NPC-treated rats showed statistically significant weight increases in comparison to the dead NPC mutants starting at day 50 of age (p < 0.05) and to untreated mutants starting at day 50 (*p < 0.05). Mutant rats that received live NPCs did not see statistical differences compared to untreated normal rats (p > 0.05). Data shown are mean ± SEM; asterisks indicate significant weight differences between live NPC and dead NPC mutants or non-treatment mutants (Repeated Measures ANOVA; Tukey's Post-Hoc Test)

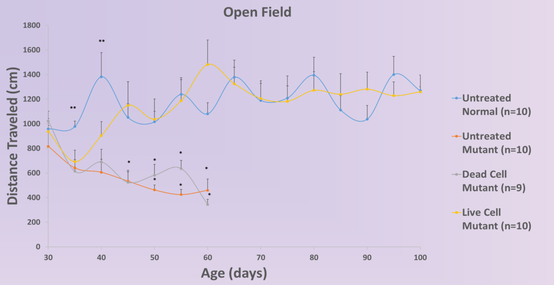

Figure 2. Open field testing of live NPC-treated mutants (n=10), dead NPC-treated mutants (n=9), non-treated mutants (n=10) and untreated normal rats (n=10) from 30 days of age until age of sacrifice. Statistical significant was found across treatments (F=13.46, p < 0.01). The live NPC-treated mutants showed a statistically significant drop in motor activity (compared to normal controls) starting at day 35 (p<0.05). However, by day 45, there was a rebound in open field activities from live NPC mutants that were statistically similar to their normal siblings (p>0.05). This rebound continued until the end of the experiment. In contrast, dead NPC mutants showed significant declines in motor activity by day 45 and were statistically different to live NPC mutants (p<0.05). This was also found in the untreated mutants starting at day 45 and continuing until the end of the experiment (p<0.05). Data shown are mean±SEM; single asterisks indicate significant difference in fall latency between live NPC and dead NPC mutants or non-treatment mutants, and double asterisks indicate differences between live NPC mutants and untreated normal rats (Repeated Measures ANOVA; Tukey's Post-Hoc Test).

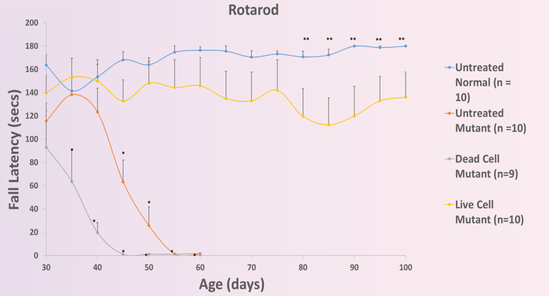

Figure 3. Rotarod testing of live NPC-treated mutants (n=10), dead NPC treated-mutants (n=9), untreated mutants (n=10) and untreated normal rats (n=10) starting at 30 days. Statistical significant was found across treatments (F=25.03, p<0.001). Dead NPC treatment showed significant differences compared to live NPC treatments (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), but starting at day 80 their scores became statistically different (p<0.05). Data shown are mean ± SEM; single asterisks indicate significant difference in fall latency between live NPC and dead NPC mutants or non-treatment mutants, and two asterisks indicate differences between live NPC mutants and untreated normal rats (Repeated Measures ANOVA; Tukey's Post-Hoc Test).

Conclusions

- Implantation of live neural progenitor cells (NPCs) reversed ataxia symptoms and increased longevity, reverting the sHW rats to normal levels of weight gain, rotarod, and motor activity. No mutant rats died after NPC transplantation until histology at 100 days of age (while all mutant rats that received dead NPC would have succumbed few days after perfusion at 60 days).
- The implanted dead NPC group showed similar decline and lack of motor skills to that of untreated mutants, confirming the lack of any paracrine effects.
- Few surviving neural progenitor cells were observed 60 days post-transplantation. but did offer neuroprotective effects close to the injection site suggesting some perfusable substance may have played a vital role in Purkinje cell neuroprotection.
- Injection of NPCs resulted in significant increase in the number of the recipients' own Purkinje cells. The proteomic analysis that discovered a number of neuroprotective factors secreted by the cells may provide insight into the mechanism of action. Paracrine stimulation of the proliferation of the rat Purkinje cells is a likely cause of improvement in these animals.

Works Cited

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Acknowledgements

We would like to thank the CSUN vivarium staff for caring for the animals, and funding for this work was provided by CSUN.