J Appl Genet 48(3), 2007, pp. 269–272

Short communication

Neural stem cell implantation extends life in Niemann-Pick C1 mice

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Abstract. In order to evaluate the phenotypic effects of implanted neural stem cells (NSCs) in the mouse model of Niemann-Pick C (NPC) disease, we injected a well-characterized clone of murine NSCs into the cerebella of neonatal *Npc1*$^{-/-}$ and control mice. The implanted cells survived and were abundant in some regions of the cerebellum. Life span was lengthened in NPC mice with the implanted NSCs. However, the rate of weight gain and subsequent weight loss, resulting from neurodegeneration, was not significantly different from un-injected controls. Ataxia was measured by Rota-Rod performance. The overall rate of decline in time on the Rota-Rod was not significantly slowed down. Thus, in this small group of NPC mice, a single administration in the neonatal period of the NSCs (which were not engineered to over-express the missing gene and not directed into the parenchyma) was only partially therapeutic.

Keywords: cerebellum, disease, neurodegeneration, neurons, neuroprotection, Niemann-Pick, Purkinje cells, stem cells, stem cell transplantation, type C.

In Niemann-Pick type C (NPC) disease, cerebellar pathology is an early feature with cholesterol accumulation in Purkinje neurons (PNs) in NPC1 mice at postnatal day 9 (Reid et al. 2004) and activation of microglia in the cerebellum at 2 weeks (Baudry et al. 2003). Neural stem cells (NSCs) derived from embryonic day 16 (E16) *Npc1*$^{-/-}$ mice have been shown to be defective in self-replication, possibly related to elevated p38 MAP kinases (Yang et al. 2006). However, we are not aware of any experiments with implanting normal NSCs into *Npc1*$^{-/-}$ mice. Given the success of NSC transplantation in complementing genetic defects (Snyder et al. 1995; Taylor et al. 1997; Lacorazza et al. 1996), replacing glia (Yandava et al. 1999; Taylor et al. 2006), and in replacing neurons and/or rescuing endangered host neurons in a wide range of cell-autonomous degenerative diseases – including those in the cerebellum (Rosario et al. 1997; Li et al. 2006), cortex (Snyder et al. 1997; Park et al. 2002) and substantia nigra (Ourednik et al. 2002) – we implanted a stable clonal population of engraftable *lacZ*-expressing murine NSCs into the cerebella of neonatal *Npc1*$^{-/-}$ mice, a model of a cell non-autonomous defect. We assessed whether: (1) they could survive in the developing cerebellum to adulthood, and (2) what effect, if any, they might have on extending life span, preventing weight loss, or correcting ataxia in affected mice.

To model the behavior of NSCs, we used a well-characterized, stable, clonal population of engraftable *lacZ*-expressing murine NSCs from clone C17.2. Thus we eliminated confounders, such as variability in engraftment, migration, differentiation, and cell number, health, and survival. NSCs from clone C17.2, isolated initially from neonatal mouse cerebellum (external germinal layer), were maintained as monolayers in DMEM medium (Life Technologies, UK) with 10% fetal

Received: December 12, 2006. Accepted: January 22, 2007.
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bovine serum-containing medium and 5% horse serum (Snyder et al. 1992). They are *lacZ* and *neo* positive. For the transplant experiment, we did not use any cultures that were carried out or passaged for more than 8 weeks.

Entire litters of *BALB/c Npc1*$^{-/-}$ mice were implanted with the NSCs on day 1 after birth. Such litters contained animals of 3 genotypes: homozygous, heterozygous, and wild-type. The latter 2 unaffected genotypes served as controls for the affected homozygous genotype. They were anesthetized on ice and their heads placed over a bright fiber-optic light. NSCs (2 μL of 2×10^7 cells mL^{-1}, i.e. 4×10^4 cells) were injected into each hemisphere of the cerebella, within the external granular layer. Another injection was then done into the midline vermis. Once revived, the mice were returned to maternal care. Food (with 10% fat) and water was available *ad libitum*. Genotyping was performed at 21 days and 16% of the mice were determined to be *Npc1*$^{-/-}$ (5 females and 3 males). Four male and four female un-injected *Npc1*$^{-/-}$ mice were used as controls.

Brains of 5 *Npc1*$^{-/-}$ mice were obtained at various ages up to 63 days, while *Npc1*$^{+/-}$ and *Npc1*$^{+/+}$ injected controls were studied up to 94 days. They were fixed in 4% fresh paraformaldehyde with 2 mM $MgCl_2$ and 2 mM EDTA. The cerebellum was sliced into small cubes (approximately 2–3 mm cubed). After brief rinsing in PBS with 2 mM $MgCl_2$ and 2 mM EDTA, they were soaked for 10 min in this solution with 0.01% deoxycholate and 0.02% NP40. They were then incubated overnight (ca. 15 h) in a 37°C water bath with X-gal solution (50 μg mL^{-1} X-gal in 5% dimethyl formamide, 95% PBS, 4.75 mM K-ferrocyanide, 4.75 mM K-ferricyanide) to stain histochemically for β-galactosidase activity. Ten 10-μm sections were cut, counterstained with nuclear-fast red, and visualized under bright-field microscopy.

The implanted NSCs survived and migrated to the cerebella of mice as originally described (Rosario et al. 1997; Snyder et al. 1992). They persisted at least up to the age of 63 days in *Npc1*$^{-/-}$ mice. Most of the β-galactosidase-positive cells (recognized as Xgal+ cells) were granule cells (based on size, morphology, and location within the internal granule layer). The staining was not artefactual, e.g. PNs were not Xgal-positive. Hence no background Xgal staining was detected (Sanchez-Ramos et al. 2000). Furthermore, few or none of the NSCs differentiated into PNs based on histochemical criteria, although it is possible that the transgene may have been down-regulated. PNs degenerated as in non-implanted *Npc1*$^{-/-}$ mice (Figure 1). The degree of implantation was similar in *Npc1*$^{-/-}$, *Npc1*$^{+/-}$ and *Npc1*$^{+/+}$ mice (data not shown).

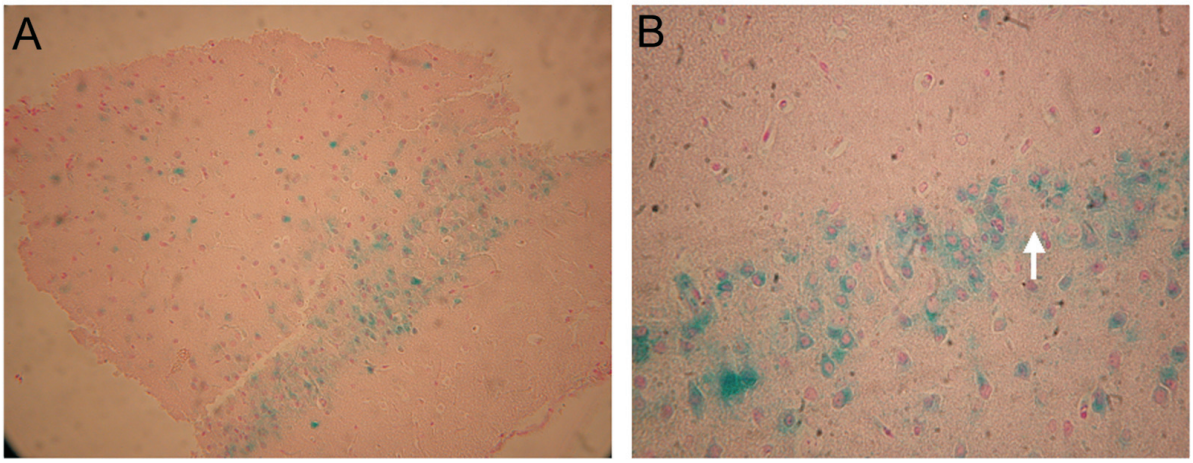

Figure 1. Slice of cerebellum from a female *Npc1*$^{-/-}$ mouse, aged 63 days. The cells stained blue are injected NSCs that are positive for beta-galactosidase. (B) is a close-up of (A). The arrow indicates a putative degenerated Purkinje cell.

The average lifespan for the implanted *Npc1*$^{-/-}$ mice that were not sacrificed for samples, was lengthened: 90.6 ± 5.8 vs. 73.2 ± 8.1, $P \leq 0.003$ (Table 1). Implanted *Npc1*$^{-/-}$ and control *Npc1*$^{-/-}$ mice showed the same rate of weight gain until days 41–46, when both groups began to loose weight (data not shown). The weights of the implanted mice stayed mostly at or below the maximum weight of the controls.

To measure the phenotypic effect of the NSCs, weights and scores on the Rota-Rod machine (Ugo Basile, NY, USA) of the mice were taken, starting at 4 weeks of age. Mice were tested weekly on the

Table 1. Survival of control *Npc1*$^{-/-}$ mice compared to *Npc1*$^{-/-}$ mice injected with NSCs (difference significant at $P \leq 0.003$). SD = standard deviation

Control		Injected	
No. of mice	Day of death	No. of mice	Day of death
1	61	1	84
1	64	2	94
1	65		
2	66		
1	69		
1	72		
1	73		
1	79		
1	80		
1	81		
3	83		
Mean ± SD	73.2 ± 81	Mean ± SD	90.6 ± 5.8

Rota-Rod. They were given 2 trials to stay on a constant-speed Rota-Rod (~25 rpm), and the 2 measurements were averaged. The results from the Rota-Rod test were erratic. Initially the implanted mice had higher Rota-Rod scores on this test of motor coordination, but they became less coordinated than the *Npc1*$^{-/-}$ controls at older ages. The rate of decline was not slowed by NSC implants in either sex (data not shown).

Our initial use of murine NSCs from clone C17.2 for cerebellar transplantation seemed reasonable, since they have high rates of differentiation into granule cell neurons (Rosario et al. 1997), which also seem to have a 'chaperone' effect on adjacent cells, such as PNs (Li et al. 2006; Ourednik et al. 2002). We reasoned that these NSCs could rescue *Npc1*$^{-/-}$ mice by developing into non-mutant neurons and/or supportive glia. There are considerable data suggesting that glia play a role in the pathogenesis of this disorder. In the primate brain, the NPC1 protein was detected predominantly in perisynaptic astrocytic processes surrounding axon terminals and dendrites (Patel et al. 1999). Many data suggest that abnormal NPC1 function in glia influence lipid trafficking between neurons and glial cells (Ong et al. 2001). *Npc1*$^{-/-}$ neurons show a deficiency of the cholesterol-synthesis regulator, i.e. sterol regulatory element binding protein 1 (SREBP1; Ong et al. 2000), which could be secondary to the altered delivery of cholesterol. It is likely that receptor recycling, synaptic vesicle dynamics, neuronal plasticity, and maintenance of the integrity of the myelin sheath, may be critically dependent on intrinsic sterol recycling between glia and neurons, a paracrine trophic effect. NPC1 and NPC2 might be key regulators of this recycling process.

In this small group of mice, NSCs implanted into interstitial regions surrounding the cerebellum of neonatal NPC mice were partially therapeutic. The implanted mice lived longer, though weight loss and Rota-Rod performance were unaffected. This observation raises the question as to the mechanism by which the NSCs exerted even this partial effect. Although NSCs survived and migrated, they did not appear to differentiate into significant numbers of PNs nor did they appear to prevent PN loss. Of course NPC disease is multisystemic and its nutritional status, muscle mass, and even Rota-Rod function are determined by many factors beyond the cerebellum and the PNs. One explanation for only a partial impact and insight into improving the outcome, would be to insure a greater number of NSCs making direct cell-cell contact with PNs. Li et al. (2006) recently determined that NSCs implanted into the parenchyma of the external germinal layer of the newborn *nervous* PN degeneration mouse mutant go onto making direct cell-cell contact with PNs (as opposed to simply being injected or residing in the interstitial spaces or meninges), correlating with the rescue of host PNs. The number of cells that make contact with one another correlates directly to the number of neurons rescued (to the point of preserving the entire Purkinje cell layer) and the more neurons were rescued, the better Rota-Rod function was. It is possible that NSC implants *in utero* or in other areas besides the cerebellum may also yield a greater therapeutic effect.

Another explanation for these results, however, is less technical and more fundamental to NPC disease. Ko et al. (2005) recently used chimeras of *Npc1*$^{-/-}$ and normal cells and found that *Npc1*$^{-/-}$ Purkinje cell death was autonomous. PN loss occurred even when wild-type Bergmann glia were in the immediate vicinity and wild-type PNs survived even if surrounded by mutant glia. Phagocytic microglia appeared as PNs died. It is possible that PNs will be difficult to rescue. And this conundrum is compounded by the fact that the window for PN neurogenesis has passed and hence cues are not available to the NSCs for PN replacement.

Acknowledgments. We thank Jessica McVey for secretarial assistance. This research was funded by the NIH grant RO1-EB00343 and the Holsclaw Family Professorship (to RPE).

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