Executive Summary: Canavan's disease [CD] is a severe leukodystrophy that affects children and is characterized by a progressive spongy degeneration of white matter. The Central Nervous System (CNS) shows substantial vacuolization and considerable demyelination. Clinical symptoms include megalencephaly, optic atrophy, severe psychomotor retardation and generalized seizures. CD results from lack of a single enzyme namely aspartoacylase [N-acetyl-L-aspartate amidohydrolase] which catalyses deacetylation of an abundant [5–10mM] amino acid derivative N-acetyl aspartic acid [NAA] to aspartic acid and acetate. The latter is thought to contribute to myelin formation. An absence of functional aspartoacylase results in N-Aspartyl aciduria i.e. excretion of NAA in the urine.

CD is a genetic disorder prevalent among Ashkenazi Jews (affecting one in 6400-13500 people) for which an effective treatment is yet to be developed. The aspartoacylase gene has been successfully cloned and its mutations have been extensively studied but there is no clear correlation between severity of the disease and the type of mutations. An animal model-the CD mouse was artificially engineered by deleting 10 base pair (bp) in exon IV of the murine AspA gene by homologous recombination in embryonic stem cells and presents an ideal model to develop gene therapy strategies to be applied to human patients.

Several studies have attempted to alleviate the major molecular symptoms of CD - accumulated NAA and water and vacuolization of the white matter in CD patients using acetazolamide, lithium citrate and acetate supplementation. Gene replacement strategies using non viral and viral formulations have also been attempted to correct the enzyme deficiencies and have shown varying degrees of improvement in disease symptoms. Recently some labs including ours have identified several novel vectors that are significantly better in transducing the CNS. The viral gene replacement studies had used AAV2 as their gene delivery vehicle; so it follows that the use of a virus that can transduce the CNS better may lead to a further improvement in the disease symptoms. The primary goal of this proposal is to improve on the existing gene delivery strategies to limit the progress of neurodegeneration.

We focus on maximizing efficiency of gene delivery to the CNS using the AAV9 capsid by two aims. The **first aim** includes defining a window of gene delivery that is sufficient to correct the disease phenotype and the **second aim** establishing the most efficient route of administration.

Description of Experiments:

We reported our results pertaining to the first aim i.e. establishing a time window of vector delivery in our semiannual report. To follow through with our second aim of optimizing the delivery method, we now delivered the virus intracerebroventricularly (ICV) at postnatal Day 1. We performed similar studies as reported in our earlier report including recording weights at regular intervals to plot growth curve and performing motor function tests at specified ages of 1 month, 3 month and 6 months. These tests included the rotarod test at a fixed speed of 3 rpm for a maximum of 3 minutes, accelerated speed of 4-40 rpm in 5mins and a balance beam test in which mice were expected to balance themselves on a wooden plank for a maximum of 3 minutes.

Results:

1. Single intravenous (IV) and intracerebroventricular (ICV) injections of AAV9-AspA rescue the lethal phenotype and improve the weight gain profile of AspA$^{-/-}$ mice.

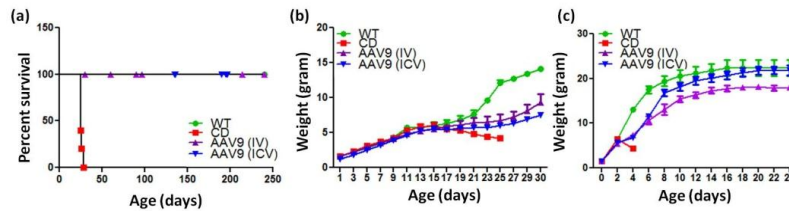The injected animals were monitored for survival and we found that all of the untreated animals died

Figure 1. Improvement of survival and growth in injected animals. CD pups (n=7) were injected with 4X10^{11} viral particles intravenously on postnatal Day1 and with 2X10^{10} viral particles intracerebroventricularly and monitored for survival (a) and weighed at regular intervals for early time point (in days) (b) and later time points in weeks (c)

between the ages of 26-28 days whereas our treated animals are still surviving to beyond an age of 6 months. (Fig 1a) These were also weighed regularly and their growth profile was monitored biweekly (Fig 1b). The animals showed a consistent increase in weight when injected at postnatal day 1 by both IV and ICV injections at the early and late time points. Although both sets of injected animals rapidly gained weight after the 6th week of their life; ICV injected animals showed a better weight gain and were closer in weight to the WT animals than the IV injected animals.

2. Delivery of AAV9-AspA by IV and ICV injections rescues vestibulomotor defects in CD mice.

The injected cohorts were tested for vestibulomotor abilities by their performance on a rotarod moving at fixed and accelerated speeds at different ages of 1 month, 3 months and 6 months (Fig 2). The 1 month animals were compared to untreated animal 28 days of age. The 1 month old animals were found perform significantly better than untreated animals on the rotarod moving at a fixed speed of 3rpm (Fig 2a) when injected at
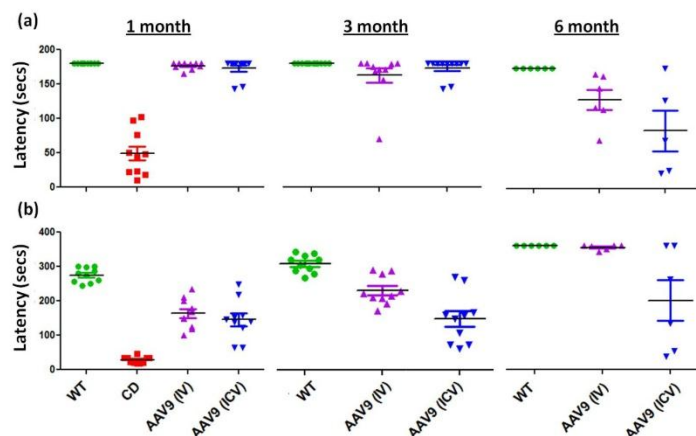

Figure 2. Improvement of motor coordination in injected animals. CD pups (n=10) were injected with 4X10^{11} viral particles intravenously on postnatal Day1 and with 2X10^{10} viral particles intracerebroventricularly and monitored for their performance on the rotarod moving at fixed speed of 3rpm (a) and accelerated speed from 4-40 rpm in 5mins (b)

postnatal day 1 by both IV and ICV injections (p<0.001) and there was no significant difference between the injected groups. On the accelerated rotarod (Fig 2b) too, both the sets of injected

animals performed significantly better (p<0.001) than the untreated animals. At 3 months of age, the IV injected animals managed to catch up in their performance with all cohorts uniformly performing as well as the WT controls (p>0.05) but the ICV animals did not do as well as the WT animals (p>0.01). At 6 months of age, the IV injected animals were still as good as the WT controls (p>0.05) but the ICV injected animals performed pretty badly being significantly different in their performance from the wildtype animals (p<0.01).

3. IV and ICV injections of AAV9-AspA improve the balance and coordinated motor functions in the disease model. To examine the animals for the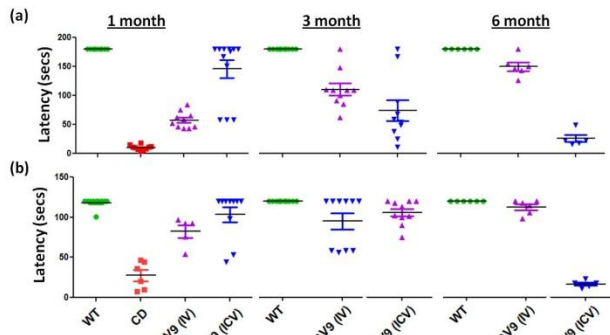ir balance and muscle strength we performed the balance beam and the inverted screen tests respectively. On the balance beam test (fig 3a) at 1 month, the treated animals performed significantly better than the untreated animals with the ICV injected animals (p<0.001) outperforming the IV injected animals (p<0.01). While the ICV injected animals grew in age, their abilities to hold onto the inverted screen seemed to diminish while that of the IV injected animals seemed to improve. At the 3 month time point both sets

Figure 3. Improvement of balance and muscle strength in injected animals. CD pups (n=10) were injected with 4X10^{11} viral particles intravenously on postnatal Day1 and with 2X10^{10} viral particles intracerebroventricularly and monitored for their ability to stay on a balance beam (a) and an inverted screen (b).

of injected animals did pretty well where the IV injected animals performed similar to the Wildtype animals (p>0.05) but the ICV injected animals were not as good (p<0.01). At the 6 month time point the ICV injected animals were not able to stay on the inverted screen at all since they also showed signs of hind limb paralysis whereas the IV injected animals were able to hold on to the inverted screen.

MRI studies show a decrease in accumulation of water in the brain of AspA$^{-/-}$ mice. We also conducted MRI/MRS imaging to evaluate the extent of water accumulation in the brains of living animals (Fig 4) and found that in contrast to the wildtype mice, brains from homozygous knock-out mice show high signal intensity in the several regions of the brain and indicates a diffuse accumulation of water. The MRI signal intensity is abnormally high in the thalamus (mainly midbrain) and brain stem of the homozygous knockout mice, indicative of high water content which mirrors that of MRIs performed on human CD patients. In contrast to MRI of the untreated CD mice, treatment with rAAV-hASPA reduces the hyperintensity indicative of water accumulation in the subcortical regions, which includes the striatum, thalamus and subthalamus. Moreover the images reveal that single intravenous injection of rAAV-hAspA in CD mice partially normalizes but does not reverse the abnormal hyperintensity observed throughout the various brain regions. It appears that the ICV injections reduce accumulation of

water to a greater extent than the IV treated animals. Even at an age of 1 year post injection, MRI studies showed hyperintensity in IV injected animals to be as low as found in the 2 month old animals (data not shown).

Figure 4. Decrease of accumulated water in the brain of injected animals. CD pups were injected with $4X10^{11}$ viral particles intravenously and with $2X10^{10}$ viral particles intracerebroventricularly on postnatal Day1 and monitored for water content in their brain by T2 weighted Magnetic Resonance Imaging (MRI) and treated animals show a decrease in the hypointense signal. Anatomical images were acquired using a fast spin-echo sequence (RARE)

Conclusions: The tests for the ICV injected animals showed that these animals decline in motor abilities in the later stage of their lives. The animals develop hind limb paralysis close to 6 months of age whereas the onset is much delayed in the IV injected animals appearing at around 8 months of age. This observation suggests that the pathology of the Canavan's disease might not be restricted to the nervous system but the peripheral organs like kidneys, eyes and muscles might also be involved. Hence this would make it even more important to carefully consider the choice of vectors as well as means of delivery for therapeutic benefit to patients. Thus far, we have met with success in designing and carrying out both of the aims designated in the grant namely establishing a time window for treatment in the first six months of the grant and exploring the feasibility of different delivery methods in the later 6 months of the estimated time of 1 year. Our collective results indicate that delivery of the therapeutic vector before day 14 seems to be effective in alleviating disease symptoms indicating the efficacy of the therapeutic vector. This also indicates that since postnatal Day 14 also marks the start of myelination; delivery of our cassette aids in formation of myelin and also supports the existing theory that aspartoacylase derived acetate may well be involved in myelination. The beneficial effects on ICV injections on the weight gain profile of the animals and reduction in water accumulation combined with the remarkable rescue in motor abilities of the IV injected animals leads us to speculate that a combination injection that involved both means of deliveries might prove to have significant benefits to the gene therapy of these CD animals.
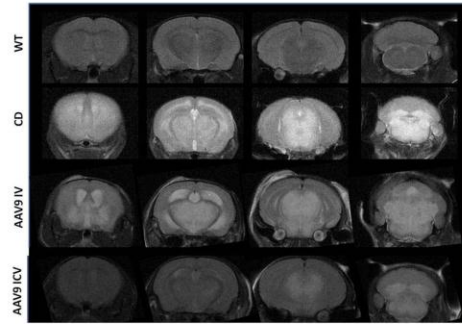
Publications: Manuscript in preparation.