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Research report

Effects of AAV-2-mediated aspartoacylase gene transfer in the tremor rat model of Canavan disease

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Abstract

The tremor rat is a spontaneous epilepsy model with a seizure phenotype caused by a deletion in the aspartoacylase (*ASPA*) gene. The absence of *ASPA* expression in these animals results in undetectable levels of enzyme activity and the accumulation of the substrate *N*-acetyl-aspartate (NAA) in brain, leading to generalized myelin vacuolation and severe motor and cognitive impairment. In support of human gene therapy for CD, recombinant adeno-associated viral vector (AAV-2) expressing *ASPA* was stereotactically delivered to the tremor rat brain and effects on the mutant phenotype were measured. AAV–*ASPA* gene transfer resulted in elevated aspartoacylase bioactivity compared to untreated mutant animals and elicited a significant decrease in the pathologically elevated whole-brain NAA levels. Assessment of motor function via quantitative rotorod testing demonstrated that rats injected with AAV–*ASPA* significantly improved on tests of balance and coordinated locomotion compared to animals receiving control vectors. This study provides evidence that AAV-2-mediated aspartoacylase gene transfer to the brain improves biochemical and behavioral deficits in tremor rat mutants (*tm*/*tm*) and supports the rationale of human gene transfer for Canavan disease.
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Theme: Disorders of the nervous system
Topic: Genetic models; degenerative disease

Keywords: Canavan; Leukodystrophy; Tremor rat; Aspartoacylase; NAA; Gene therapy

1. Introduction

Canavan disease is an autosomal recessive neurogenetic disorder caused by mutations or deletions in the aspartoacylase gene (*ASPA*) [17], which results in loss of *ASPA* enzyme function [25,26] and accumulation of its substrate *N*-acetyl-aspartate (NAA) in neural cells, cerebrospinal fluid (CSF), and brain interstitial fluid. The tremor rat has a large inactivating deletion spanning the aspartoacylase (*ASPA*) coding region [18] and thus represents a naturally occurring model of Canavan disease. As with Canavan disease, deletion of *ASPA* and the associated elevation in brain NAA lead to changes in white matter morphology, and behavioral deficits such as akinesia and loss of normal motor coordination and balance. Pharmacological approaches targeted to affected biochemical pathways

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have not been effective in alleviating the neurological deficits associated with CD [20]. Therefore we have focused on the development of gene transfer strategies to safely and efficiently provide functional aspartoacylase activity to the brain of CD patients, thereby targeting the primary causative pathological mechanism of this disease [13,21].

The monogenetic nature of CD combined with the absence of effective treatments supports the rationale of aspartoacylase gene transfer to the brain. NAA is grossly elevated in CD [25] and although its function is not well understood, abnormal accumulation of this metabolite remains the primary symptomatic feature of Canavan disease. NAA causes seizure activity when administered in high doses to normal rats, an effect that is accentuated in tremor rats which have chronically elevated NAA [2]. The loss of normal NAA catabolism in CD has been hypothesized to limit the supply of acetate for lipid synthesis [7,8,32] and is also thought to influence mitochondrial function [11,35] and cellular energetics. The role of NAA catabolism in myelin formation is still unclear. Despite the enigmatic nature of NAA function in normal cell metabolism, production of NAA is known to occur primarily in neurons with a very high intraneuronal–interstitial concentration gradient [40,42]. NAA may be involved in H_2O flux between neurons and oligodendrocytes, with hydrolysis of the osmolyte via aspartoacylase occurring at the cell membrane of oligodendrocytes [6,43]. In CD the intercompartmental cycling of NAA in the absence of breakdown by aspartoacylase is thought to result in a loss of osmotic control and the swelling of astrocytes with formation of spongiform vacuoles [1,44].

A definitive physiological role for *ASPA* in the brain is currently lacking, but the correction of the CD phenotype at a symptomatic stage of disease via gene transfer is a possibility that requires evaluation. AAV-2 is well characterized and widely used as a vector system for the delivery of genetic material to the brain [19,34,36,38,45]. One advantage is the relative absence of immunological responses following its administration and the ability to sustain long-term gene expression in vivo [14,22,24,29]. AAV-2 possesses characteristics of efficacy and safety that offer advantages over other currently available viral vector systems, particularly for clinical applications. Our working hypothesis for the current study was that AAV-2-mediated aspartoacylase gene transfer into neurons in the tremor rat brain results in the breakdown of NAA in the compartment of synthesis, thereby reducing the efflux of metabolite into other compartments and promoting a subsequent decrease in downstream pathology.

Molecular and physiological characterization of the tremor rat (*tm*/*tm*) indicates an absence of aspartoacylase expression as well as accumulation of NAA in brain tissue [2,18]. Rats heterozygous for the aspartoacylase deletion have a relatively normal phenotype, although some go on to develop absence-like seizures as adults. A previous study documented behavioral deficits in tremor rats using the rotorod assay of coordinated locomotion [3], thereby providing a quantitative assessment of phenotype following gene transfer. This assay along with measures of enzyme bioactivity and tissue NAA content were chosen to evaluate the effect of AAV-mediated aspartoacylase gene transfer in tremor rats.

2. Materials and methods

2.1. AAV-2 vector construction

The AAV-2 expression cassette was optimized for neuronal gene transfer with the use of the neuron-specific enolase promoter (*NSE*, provided by J.G. Sutcliffe, Scripps Research Institute) [19,30,31,36,45] and the human aspartoacylase gene (*ASPA*, provided by R. Matalon, University of Texas) [17]. The *ASPA* expression cassette was packaged into recombinant AAV-2 using the pDG helper plasmid system as previously described [12]. Woodchuck post-regulatory elements (WPRE) and bovine growth hormone polyA sequences were utilized to boost gene expression, as previously described [45]. Vectors were DNAse and Proteinase K treated before being titered by quantitative real time PCR (7700, Applied Biosystems) using CYBR Green® to detect linear amplification of a region within the common WPRE sequence, a modified version of the method of Clark et al. [10].

2.2. Gene delivery to tremor mutant cohorts

Three cohorts of rats were used for analyses presented in this study. Cohort 1 was used for biochemical and behavioral analyses, and Cohorts 2 and 3 were used for immunocytochemical analyses. Tremor mutant availability was limited, and both male and female mutants were studied. Cohorts 1 and 3 were males, and Cohort 2 animals were female to both optimize animal use and limit potential variability.

2.3. Gene delivery and behavioral and biochemistry assay time points: Cohort 1

A cohort of 12 male tremor rats received intracranial gene delivery at 30 weeks of age. Behavioral testing was performed on these animals 10 weeks later (40 weeks of age) before tissue collection for biochemical assays. Intracranial gene delivery was performed under 2% isoflurane inhalation anesthesia using standard stereotactic microsurgical techniques [10]. Male tremor rats received bilateral microinjections of either AAV–*ASPA* (n = 6) or AAV–*GFP* (control, n = 6) in the caudate (AP −0.92 mm (from bregma), ML ± 3.5 mm (from midline), DV −3.8 mm (from dura), and thalamus (AP −2.80 mm (from bregma), ML ± 3.0 mm (from midline), DV −3.8 mm

(from dura). The total dose delivered to each animal was 3.2×10^{10} vector genomes, spread evenly across each site in four injections of 4 μL each, with an infusion rate of 0.2 μL/min. We also studied naïve age-matched male wild-type, heterozygous, and tremor for comparison in biochemical analyses. A follow-up period of 10 weeks was used after which behavioral testing was performed on all male tremor rats. At 40 weeks of age all rats were humanely euthanized (Euthanasol®, 1 mL/kg i.p.) following Institutional Guidelines. The cranium was immediately opened to allow removal of fresh brain tissue for dissection and immediate freezing as described below.

2.4. Gene delivery and immunocytochemistry assay time points: Cohorts 2 and 3

Cohort 2: A cohort of nine female tremor rats received intracranial gene delivery at 30 weeks of age, before transcardial perfusion at 40 weeks of age. Female tremor rats received unilateral injections of AAV–*ASPA* (*tm*/*tm*, $n = 3$), AAV–*GFP* (*tm*/*tm*, $n = 3$), as well as PBS vehicle (*tm*/*tm*, $n = 3$). Gene or vehicle delivery was undertaken as described above for males, with injections to the left hemisphere only. The total dose delivered to each animal was 1.6×10^{10} vector genomes, spread evenly across the two injection sites within the left hemisphere caudate and thalamus, with an infusion rate of 0.2 μL/min. Cohort 3: A cohort of two male tremor rats received intracranial gene delivery at 16 weeks of age, before transcardial perfusion at 19 weeks of age. One male tremor rat received bilateral injections of AAV–*ASPA* and another received AAV–*GFP*. The injection coordinates were identical to those used in Cohort 1. The total dose delivered to each animal was 4.8×10^{9} vector genomes split evenly amongst the four injection sites.

2.5. Immunohistochemistry

Brains from Cohort 2 were transcardially perfused with 1× PBS (pH 7.4) followed by 4% paraformaldehyde (pH 7.4), then cryosectioned at 14 μm and mounted directly onto coated glass slides. The presence of exogenous aspartoacylase protein in rats treated with AAV–*ASPA* was confirmed in fixed tissue using a polyclonal antibody to aspartoacylase, courtesy of Dr. M.A.A. Namboodiri. Primary antibody was diluted 1:1000 in buffer (1× PBS, 0.2% Triton-X 100, 1% normal goat serum) and incubated with sections overnight at ambient temperature, then washed 3× for 5 min in 1× PBS with 0.2% Triton-X 100. Sections were then incubated for 2 h at RT with CY3-conjugated secondary antibodies (Goat anti-rabbit, Jackson Immunochemical Laboratories). Finally, sections were washed 3 × 10 min in PBS–Triton-X, slide mounted and coverslipped in 50% glycerol/0.1 M phosphate buffer. A qualitative assessment of aspartoacylase distribution was undertaken. Brains from Cohort 3 were post-fixed overnight in 4% PFA followed by cryopreservation in 10–30% sucrose solutions and were then sectioned at 40 μm. Brain sections from the GFP-injected animal were immediately placed in 0.1 M phosphate buffer prior to processing, then permeabilized with 3 × 15 min washes with 1× PBS containing 0.1% Triton-X 100, and then incubated with either mouse anti-NeuN (Chemicon, 1:1000), mouse anti-GFAP (Chemicon, 1:500), or mouse anti-APC (CC-1, Oncogene, 1:500) overnight at RT. Sections were then washed 3 × 10 min in PBS–Triton-X, then incubated for 2 h at RT with CY3-conjugated secondary antibodies (Rabbit anti-mouse, Jackson Immunochemical Laboratories, 1:250). Finally, sections were washed 3 × 10 min in PBS–Triton-X, slide mounted and coverslipped in 50% glycerol/0.1 M phosphate buffer. Immunocytochemical detection of aspartoacylase was also performed as described for Cohort 2. Immunofluorescence images were obtained from a digital camera (Retiga EX, Q Imaging) connected to a UV microscope (IX70, Olympus) with IP Lab (v3.6) acquisition software.

2.6. Biochemical analyses

Non-fixed (fresh) tissue samples were obtained from discrete regions of AAV–*ASPA* (*tm*/*tm*, $n = 6$, 36 samples) and AAV–*GFP* (*tm*/*tm*, $n = 6$, 36 samples) animals for biochemical analysis, using brain dissected bilaterally from regions proximal to the striatal and thalamic injection sites (anterior caudate and thalamus) and one region between the injection sites (posterior caudate), with a total of six samples collected from each brain. A sample of the thalamus from each tremor rat that received AAV–*ASPA* or AAV–*GFP* was also taken for RT-PCR analysis. Age-matched naïve untreated wild-type Kyoto–Wistar rats (+/+, $n = 2$, 12 samples) and untreated homozygous (*tm*/*tm*, $n = 2$, 11 samples) and heterozygous (*tm*/+, $n = 2$, 12 samples) tremor rats were also studied. Procedures for aspartoacylase enzyme assays were as previously described [4]. In brief, [^{3}H]-acetate-labeled NAA was custom synthesized (20 mGy/nmol) and specific *ASPA* enzyme activity was obtained in units of nmol acetate released/mg protein/5 h. Procedures for HPLC quantification of NAA were also as previously described [41]. In brief, NAA was analyzed using two ESA 580 HPLC pumps (Chelmsford, MA) connected to a Shimadzu solvent mixer (Columbia, MD). Analytes were detected by UV absorbance at 210 nm using a Linear UVIS 204 UV detector (Linear, Reno, NV) and quantified with reference to external calibration standards using a Spectra Physics Chromjet integrator (Thermo-Separations).

2.7. Behavioral testing

Quantitative behavioral testing was undertaken with a rat rotorod system (Rotamex-4, Columbus Instruments). We used a graduated increase in the test difficulty [39] to

assess the motor impairment in treated and untreated animals. In phase 1, animals were each introduced to the stationary rotorod on five trials with a maximum duration of 120 s with 1 min separation between each trial. Following an interval of 24 h, animals were tested for five sessions on a moving rotorod at 3 rpm (phase 2) and then tested 24 h later over another five trials also at 3 rpm (phase 3), both with a maximum duration of 60 s. The maximum time balancing on the rotorod (rotorod latency) was measured for each animal for each of the three phases of the study. All animals were tested at the same terminal time point of 10 weeks post-gene transfer, prior to sacrifice.

2.8. Statistical analyses

Data were analyzed with Stata (v 7.0, Stata Corp), Statview (v5, SAS Institute), and with Prism (v 3.0 Graphpad Software). Aspartoacylase bioactivity and NAA concentration data were analyzed by non-parametric one-way analysis of variance (ANOVA, the Kruskal–Wallis test) and Student's *t* tests (assuming unequal variance). Rotorod latencies were analyzed by non-parametric one-way ANOVA, repeat measures ANOVA, and Student's *t* tests (assuming unequal variance).

3. Results

3.1. Expression of exogenous ASPA in AAV-transduced tremor rats

Transcription of AAV-delivered expression cassettes in injected tremor rats was confirmed by RT-PCR analysis of total brain RNA isolated from the thalamus, which detected mRNA encoding for the WPRE sequence element within AAV-2 expression cassettes in 11 of 12 tested animals (data not shown). The presence of exogenous ASPA protein after gene transfer was confirmed by immunocytochemistry (Fig. 1). Aspartoacylase-labeled cell bodies or processes were observed with immunocytochemistry and appeared in coronal planes anterior and posterior to the injections sites in the caudate and thalamus, appearing up to 2 mm from the injection site. Representative brain sections from naïve tremor rats and tremor rats injected with AAV–*ASPA* or AAV–*GFP* were processed with antibody to aspartoacylase, shown in Figs. 1 and 2. Intense labeling of aspartoacylase was observed in the AAV–*ASPA*-injected tremor rat thalamus (ventral anterior, posteriomedial, and posteriolateral regions) as shown in Fig. 1 but no labeling was observed in the thalami of tremor rats injected with AAV–*GFP*. No ASPA-positive

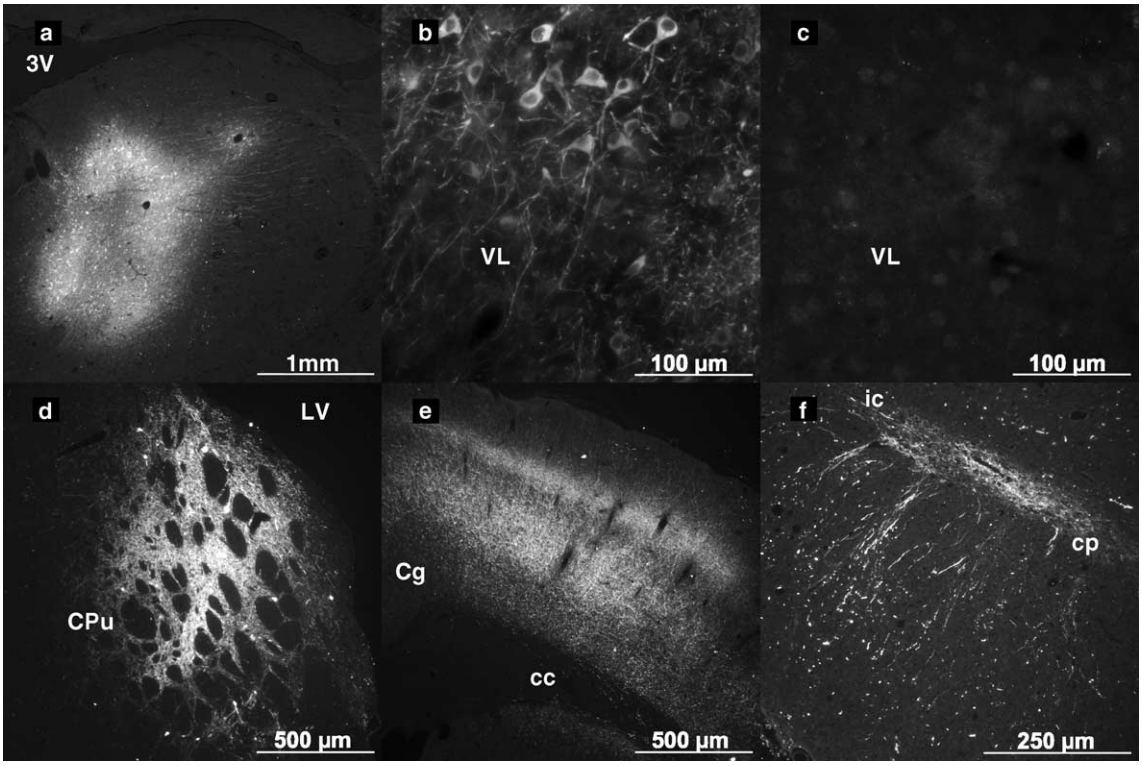

Fig. 1. Aspartoacylase expression in the tremor rat brain. Panels a and b are AAV-*ASPA* injected tremor rat brain sections, showing anti-aspartoacylase labeling of cells in the thalamus at 2× and 20× magnification, respectively. Panel c is a control 20× magnification section from a naïve tremor rat thalamus. Panel d is a 4× magnification image of the AAV–*ASPA*-injected caudate. Panel e is a 4× magnification image of the cortex showing cell processes labeled with anti-aspartoacylase. Panel f is a 10× magnification image of the cerebral peduncle and internal capsule showing cell processes labeled with anti-aspartoacylase. Abbreviations: 3V, third ventricle; VL, ventrolateral thalamic nucleus; CPu, caudate putamen; LV, lateral ventricle; Cg, cingulate cortex; cc, corpus callosum; ic, internal capsule; cp, cerebral peduncle.

cells were observed in AAV–*GFP* transduced or naïve tremor rats (Fig. 1c). ASPA-positive cell processes were observed in the thalamus, caudate, cortex, and white matter tracts of the AAV–*ASPA* transduced tremor rat brain, suggestive of protein located in axons and processes from cells transduced in the injected caudate and thalamus. Aspartoacylase-positive cell processes were observed in the anterior commissure, the corpus callosum (Fig. 1e), the external and internal capsules, and the cerebral peduncles (Fig. 1f), all regions distal to the injection sites. There was no labeling of aspartoacylase in sections from the cerebellum.

3.2. AAV2-mediated transgene expression is predominantly observed in neurons

Brain sections from the third cohort of tremor rats were examined to verify the neuronal tropism of AAV2 vectors delivered to the CNS (Fig. 2). Antibodies to NeuN (Fig. 2a and c), GFAP (Fig. 2e and f), and APC (Fig. 2h and i) were used to label neurons, astrocytes, and oligodendrocytes, respectively. As with previous studies [5,9,19,45] AAV serotype 2 vectors are neurotropic, with just a small percentage of transduced cells (~1–5%) having a glial phenotype. Fig. 2a–c show labeling with NeuN in sections containing GFP-positive cells in the thalamus. Fig. 2d–f show labeling with GFAP in sections containing GFP-positive cells in the caudate. Fig. 2g–i show labeling with APC in sections containing GFP-positive cells in the internal capsule. While the majority of transduced cells co-label with NeuN, very few GFAP or APC-positive cells were observed.

3.3. Gene transfer with AAV–ASPA restores functional ASPA bioactivity in the tremor rat

After demonstrating that ASPA was present in treated animals but absent in untreated homozygous animals, we

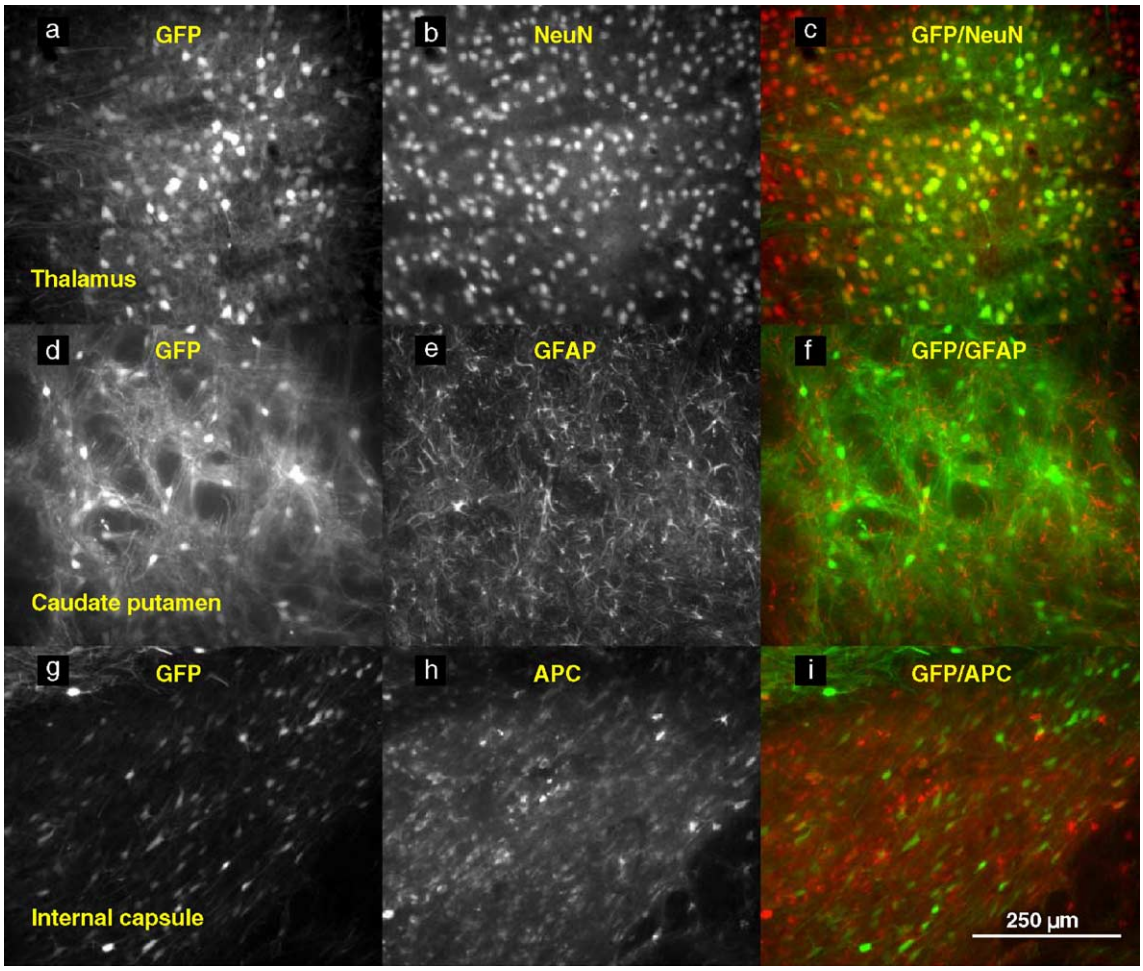

Fig. 2. Neuronal tropism of AAV serotype 2 vectors. Panels a–i show transgene expression and co-labeling with neural cell type markers in animals injected with AAV–*GFP*. Panels a–c are from the thalamus, with a showing GFP expression, b showing NeuN-positive cells (neurons), and c being an overlay of a and b showing a majority of GFP expressing cells also positive for NeuN. Panels d–f are from the caudate, with d showing GFP expression, e showing GFAP-positive cells (astrocytes), and f being an overlay of d and e showing that cells expressing GFP are not positive for GFAP. Panels g–i are from the internal capsule, with g showing GFP expression, h showing APC-positive cells (oligodendrocytes), and i being an overlay of g and h showing that cells expressing GFP are not positive for APC. All panels were from 10× magnification images, and the scale bar corresponds to 250 μm.

examined the ability of AAV-2 gene transfer to restore aspartoacylase bioactivity in tremor rat brain tissue, by assaying the release of acetate from a [^{3}H]-labeled NAA molecule as a measure of hydrolysis by functional enzyme (Table 1). The mean aspartoacylase activity levels (nmol acetate released/mg protein/5 h) assayed from tissue taken from tremor rats injected with AAV–*ASPA* (6.36 ± 1.65, mean ± SE) were ~37% of levels determined for naïve heterozygous animals (17.17 ± 1.80) providing evidence of AAV-2-mediated correction of the biochemical deficit present in tremor rats. The observed differences in aspartoacylase bioactivity between groups were statistically significant. Non-parametric one-way ANOVA (Kruskal–Wallis test) testing gave a Kruskal–Wallis statistic of 68.11 and an associated P value of <0.0001 (df = 4). Unpaired two-sided Student's t tests with the assumption of unequal variance were used to examine whether the observed differences in enzyme bioactivity levels were statistically significant at the α = 0.05 level. Assuming unequal variance, the AAV–*ASPA* vs. AAV–*GFP* comparison produced a P value of 0.0006, considered highly significant (*Fig. 3A).

3.4. Gene transfer with AAV–ASPA lowers total brain NAA levels in the tremor rat

After demonstrating the presence of functional ASPA in AAV–*ASPA*-treated animals, it was necessary to determine the effects of *ASPA* gene transfer as applied to an important biomarker that is elevated in Canavan disease, *N*-acetyl-aspartate (NAA). HPLC was used to determine NAA content of brain homogenates from untreated homozygous and heterozygous animals, wild-type control animals, and homozygous tremor animals which had been treated with AAV–*ASPA* gene transfer (Table 1).

Naïve heterozygous tremor animals (tm/+) had an approximately 19% relative increase in NAA concentration compared to wild types (+/+) and yet were relatively normal from a behavioral standpoint. Naïve tremor mutants (*tm*/*tm*) had an approximate 56% relative increase in NAA concentration compared to wild types. In contrast, the AAV–*ASPA*-treated homozygous tremor animals (*tm*/*tm*) had only 33% elevation in mean NAA concentration compared to wild types, demonstrating the effect of gene transfer on total NAA levels. In contrast, AAV–*GFP* (control)-treated homozygous tremor rats (*tm*/*tm*) had a relative 52% increase in NAA concentration compared to naïve wild types. AAV–*ASPA*-treated homozygous tremor animals (*tm*/*tm*) had a 12% elevation in mean NAA concentration compared to naïve heterozygotes, while AAV–*GFP* (control)-treated homozygous tremor rats (*tm*/*tm*) had a relative 28% increase in NAA concentration. The observed differences in NAA concentration between all groups were statistically significant. Non-parametric one-way ANOVA (Kruskal–Wallis test) testing gave a Kruskal–Wallis statistic of 20.36 and an associated P value of 0.0004 (df = 4). Unpaired two-sided Student's t tests were used to examine whether the observed differences in group mean NAA concentrations were statistically significant. Assuming unequal variance, the AAV–*ASPA* vs. AAV–*GFP* comparison produced a P value of 0.0177 (**Fig. 3B).

3.5. Gene transfer with AAV–ASPA promotes recovery of behavioral phenotype

Previous analysis of the tremor rat phenotype has highlighted a deficit in coordinated rotorod locomotor activity [3]. Non-parametric one-way ANOVA (Kruskal–Wallis test) indicated statistically significant variance between all group mean rotorod latencies for phase 3 (P = 0.0220 with df = 2). Repeat measures ANOVA was performed to analyze the variance in measures repeated for phase 2 and phase 3 time points, giving a P value of 0.0142 (F = 13.61, df = 1). Paired two-sided Student's t

Table 1
Mean regional NAA concentrations for each genotype

Genotype	Brain region								
	Ant. caudate (mean, SD, n)			Post. caudate (mean, SD, n)			Thalamus (mean, SD, n)		
Aspartoacylase bioactivity (moles acetate released/mg proteins/5 h)									
tm/*tm* AAV–*ASPA*	7.23	12.56	12	3.42	5.74	12	8.42	10.28	12
tm/*tm* AAV–*GFP*	0.02	0.05	12	0.09	0.32	12	0.39	0.77	12
tm/*tm* Naïve	0.00	0.00	4	0.00	0.00	3	0.00	0.00	4
tm/+ Naïve	13.00	6.68	4	19.25	6.18	4	19.25	4.99	4
+/+ Naïve	39.38	14.99	4	31.08	21.40	4	29.55	17.01	4
NAA concentration (mM)									
tm/*tm* AAV–*ASPA*	9.95	1.56	12	9.81	2.81	12	10.63	2.74	11
tm/*tm* AAV–*GFP*	10.07	2.02	12	12.35	3.28	12	12.40	2.35	12
tm/*tm* Naïve	8.49	1.74	4	11.44	5.22	3	15.66	4.94	4
tm/+ Naïve	5.05	2.38	4	12.24	6.58	4	9.93	1.52	4
+/+ Naïve	7.30	1.61	4	7.73	2.79	4	7.82	1.87	4

The table summarizes raw neurochemistry data by region dissected. The three areas sampled were the anterior portion of the caudate (Ant. caudate), the posterior portion of the caudate (Post. caudate), and the thalamus including the anterior ventral, posteriomedial, and posteriolateral regions (Thalamus). Fewer naïve animals were tested and naïve group means have greater associated variance.

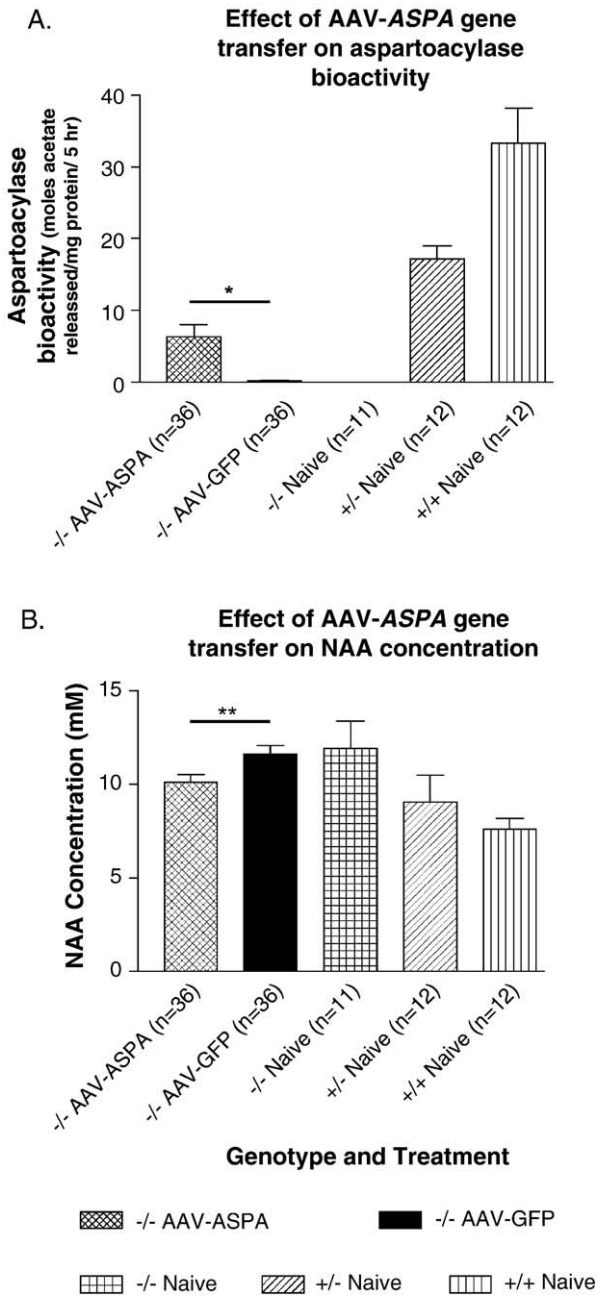

Fig. 3. Effect of AAV–*ASPA* on *N*-acetylaspartate biochemistry in rat brain. Graphs show overall mean values of bioactivity and NAA concentration for all samples tested from each genotype. (A) The mean aspartoacylase bioactivity of tissue homogenates was assessed for the tremor mutants that received gene transfer as well as for naïve age-matched *tm*/*tm*, *tm*/+, and +/+ Kyoto–Wistar rats (mean ± SEM). (B) The mean NAA concentration in tissue homogenates was assessed for the same groups and sampled areas as the bioactivity assay (mean ± SEM). Biochemical analyses could not be performed on one sample due to technical difficulties, and two dissections were not obtained on one naïve homozygous tremor rat. Table 1 presents results grouped by brain region dissected.

tests of differences between phase 2 and phase 3 results for individual treatment groups produced *P* values of 0.0616, 0.3862, and 0.5402 for AAV–*ASPA*, AAV–*GFP*, and Naïve mutant groups, respectively. We found that AAV–*ASPA*-treated tremor rats (*tm*/*tm*) showed a statistically significant improvement in coordinated locomotor function compared to the AAV–*GFP*-treated tremor rats (Fig. 4). Unpaired two-sided Student's *t* tests were used to examine whether the observed differences in group mean phase 3 rotorod latencies were statistically significant. Assuming unequal variance, the AAV–*ASPA* vs. AAV–*GFP* comparison produced a *P* value of 0.0055 (***Fig. 4). In summary, AAV–*ASPA* rats performed better with longer rotorod latencies than the injected controls for all phases of testing.

4. Discussion

In this study, AAV-2-mediated brain gene transfer of *ASPA* was performed for the first time in the tremor rat model of Canavan disease. Transduction with AAV–*ASPA* resulted in the robust expression of exogenous *ASPA* in cell bodies, almost exclusively neuronal, adjacent to the injection sites, and within axonal projections in regions distal to injection. Few cell bodies positive for aspartoacylase were observed beyond regions proximal to the caudate and thalamus. However, aspartoacylase-positive cell processes were observed in more distal brain regions suggesting protein diffusion or transport out of the cell soma. These projections transversed major white matter tracts of the brain, extending as far as the cingulated and motor cortices, and anterior and posterior boundaries of the corpus callosum. Aspartoacylase and GFP expressions in axons fibers were observed as being adjacent to oligodendrocytes in white matter tracts (Fig. 2i). Other studies have shown that infusion of AAV2 into brain regions such as the caudate [15,16] may lead to local vector uptake into cell bodies and

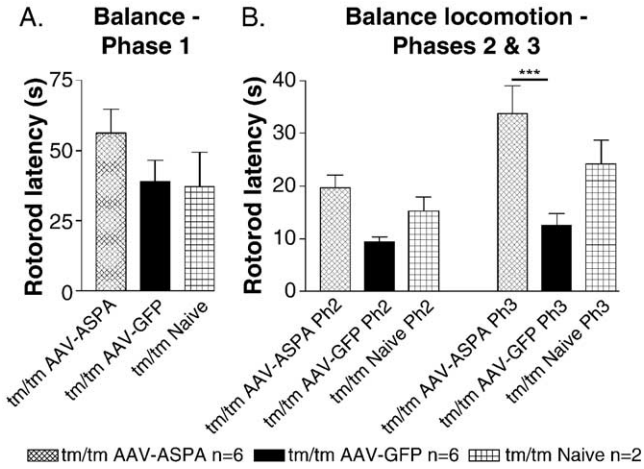

Fig. 4. Effect of AAV–*ASPA* on coordinated rotorod activity. (A) In the first phase of rotorod activity measurements, animals were placed on a stationary rotorod and were required to balance for 120 s. Bars show mean latencies from five trials with SEM. (B) In the second and third phases of rotorod activity measurements, animals were placed on a rotating cylinder and were required to walk on the rod for 60 s. Bars show mean latencies from five trials with SEM.

also into projection neurons, which retrogradely transport the vector to more distal nuclei such as the substantia nigra. Future studies will be necessary to optimize gene delivery to allow for focal and more distal cell bodies to take up AAV2 from each single injection site.

AAV-2-mediated gene expression of human *ASPA* resulted in a significant improvement in the biochemical deficit of aspartoacylase deficiency as well as behavioral improvement. We also found that AAV–*ASPA*-treated tremor animals (*tm*/*tm*) had a lower mean level of total NAA compared to untreated or animals with injected with control vector. Because it was not possible to distinguish between NAA within neurons, which normally exists at relatively high levels in healthy animals, and NAA within the interstitial space and CSF, which is elevated only in Canavan disease, variations in total NAA within a given compartment (e.g., interstitial space, glial cells) may be different from the effects in all these regions considered together. Because AAV vector was delivered primarily to the neuronal compartment at the origin of NAA synthesis, it seems reasonable to assume that the majority of the NAA decrease was in the vicinity of neurons, including the axonal compartment with oligodendrocyte and other glial attachments. The method of detection separated NAA from other metabolites such as NAAG to eliminate any confounding by contamination of the NAA peak. The rate of synthesis of NAA is markedly reduced in CD [33], possibly due to feedback inhibition [23]. The rate of NAA production and turnover could not be ascertained in this current study so it is not possible to comment on the effect of aspartoacylase gene transfer on NAA synthesis and turnover dynamics. Developments in ^{13}C NMR spectroscopy systems may allow for future experiments of acetate and NAA turnover in this model.

The selection of the thalamus and caudate as target nuclei for transduction was based on previous descriptions of regional NAA concentrations being highest in the thalami of tremor mutant brain [18] and the involvement of both the caudate and thalamus in locomotor activity related pathways. An important feature of this study was that we examined the effect of partial reconstitution of the biochemical deficits on a behavioral endpoint. The assessment of rotorod activity (Fig. 4) was used as a indirect measure of locomotor pathway function in mutant brain and the data suggest it is a sensitive method to measure locomotor changes in this particular model. Behavioral testing was only undertaken 10 weeks after gene transfer and no baseline data were available, AAV–*ASPA* injected rats appeared to perform better than the AAV–*GFP* group on the rotorod test. Analyses of rotorod latency times and test phase pass/fail data indicated that there was a biologically and statistically significant improvement in the phenotypic deficit of the tremor rat after gene transfer, despite a relatively modest drop in mean NAA concentration. The naive tremor animals performed slightly better than AAV–*GFP*-treated animals, possibly a result of the cranial surgery having an impact on mobility. Additional studies are necessary to further delineate the association between aspartoacylase deficiency and abnormal rotorod function. Naïve wild type rats are able to complete each test phase without difficulty, a result which no tremor animal obtained in this study. The seizure phenotype of this model is a potential confounder of various behavioral assays involving negative stimuli.

A previous study examined the effect of adenovirus-mediated aspartoacylase gene transfer in the tremor rat model [37], but evidence of an effect of gene transfer was limited to the study of seizure activity. A more recent study [28], also examined the application of AAV-2-mediated aspartoacylase gene transfer to the CNS of aspartoacylase knockout mice. The Canavan knockout mice exhibit a lack of functional aspartoacylase expression, delayed development, cranial abnormalities, and shortened lifespan [27], similar to the rat model. This other study provided some preliminary evidence of gene transfer of human *ASPA* resulting in improvement in the biochemical phenotype of the knockout mice. Further studies will be required to clarify the neuropathological and molecular abnormalities in these animal models of CD, including the effects of age-specific treatments. The data from this study in symptomatic tremor rats support the use of AAV-mediate aspartoacylase gene delivery to improve the biochemical and behavioral deficits associated with aspartoacylase deficiency.

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