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Antisense Oligonucleotide Reverses Leukodystrophy in Canavan Disease Mice

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Abstract

Marked elevation in the brain concentration of N-acetyl-L-aspartate (NAA) is a characteristic feature of Canavan disease, a vacuolar leukodystrophy resulting from deficiency of the oligodendroglial NAA-cleaving enzyme aspartoacylase. We now demonstrate that inhibiting NAA synthesis by intracisternal administration of a locked nucleic acid antisense oligonucleotide to young-adult aspartoacylase-deficient mice reverses their pre-existing ataxia and diminishes cerebellar and thalamic vacuolation and Purkinje cell dendritic atrophy.

Canavan disease (CD) is a vacuolar leukodystrophy caused by *Aspa* gene mutations that block expression of functional aspartoacylase, an enzyme required for catabolism of the abundant brain amino acid N-acetyl-L-aspartate (NAA).[1–3] Current therapies for CD, including dietary manipulations, lithium citrate administration, stem cell transplants, and brain intraparenchymal adeno-associated viral (AAV)-*Aspa* vector administration, have not been successful in reversing or preventing progression of CD.[4,5] The brain concentration of NAA ($[NAA_B]$) is markedly elevated in CD,[2,4] and also in aspartoacylase-deficient CD mice.[6–9] We now report that lowering $[NAA_B]$ in young-adult CD mice by administration into cisterna magna of a locked nucleic acid antisense oligonucleotide (LNA-ASO, or gapmer)[10] to knockdown expression of the neuronal NAA-synthesizing enzyme N-acetyltransferase 8-like (Nat8l)[11] reverses ataxia, Purkinje cell dendritic atrophy, and cerebellar/thalamic vacuolation.

Materials and Methods

Mice heterozygous for the Aspa nonsense mutation $Aspa^{nur7}$ were obtained from the Jackson Laboratory (JAX:008607; Bar Harbor, ME), maintained on a C57BL/6J

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Author Contributions
All of the authors contributed to conception and design of the study, drafting the text or preparing the figures, and acquisition and analysis of data.
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Potential Conflicts of Interest
Nothing to report.

background, and crossed to generate Aspa$^{nur7/nur7}$ CD mice[6,8] and age-matched Aspa$^{WT/WT}$ control mice (WT). An LNA-ASO[10] designed to knockdown Nat8l expression (Nat8l gapmer, nucleotide sequence GGCGTAGAGCAGTTGG), and a negative control LNA-ASO without known eukaryote targets (control gapmer, nucleotide sequence AACACGTCTAT ACGC), were from Qiagen (Germantown, MD). Both gapmers were constructed with a phosphorothioate backbone and 2′-O-methoxyethyl-derivatized sugar modifications.[10] Nat8l or control gapmer (0.5nmol in 5μl of artificial cerebrospinal fluid) was infused into the cisterna magna of 2-month-old CD or WT mice at 1 μl/min under isoflurane anesthesia. No CD or WT mice died during this procedure, nor, with the exception of those sacrificed for brain harvest, did any die throughout the course of this study.

Accelerated rotarod testing (starting speed 4 RPM, increasing by 1.2 RPM every 10 seconds)[8] was performed by blinded observers. For biochemical and histological studies, mice were deeply anesthetized with ketamine/xylazine and perfused with cold phosphate-buffered saline (PBS). After brain harvest, cerebella were dissected free, transected longitudinally, and cerebellar samples from each mouse were flash-frozen for Nat8l mRNA and NAA assays, or fixed in 4% paraformaldehyde in PBS, cryoprotected in 30% sucrose, and embedded in optimal cutting temperature compound prior to cryostat sectioning. Nat8l mRNA abundance was assayed by quantitative real-time polymerase chain reaction,[8] normalized to Hsp90 mRNA abundance. NAA was assayed by high-performance liquid chromatography,[12] and expressed in micromole per gram cerebellar wet weight. Cerebellar cryostat sections were cut from the sagittal cerebellar midline at 10 μm (slide mounted) or 50 μm (free floating) thickness for immunostaining. The 10μm sections were incubated with rabbit anti-PLP1 (RRID:AB_2165785, 1:200) and mouse anti-QKI-7 (CC1; RRID:AB_2173148, 1:100), and the 50μm sections were incubated with rabbit anti-calbindin (RRID: AB_868617, 1:200). For thalamic immunohistology, 10μm coronal cryostat sections were cut between Bregma −1.06mm and −1.94mm, slide mounted, incubated with rabbit anti-PLP1 (RRID:AB_2165785, 1:200) and rat monoclonal anti-GFAP (gift of Dr. Virginia Lee, University of Pennsylvania, 1:300), then incubated with fluorescently tagged secondary antibodies, counterstained with 4′,6′-diamidino-2-phenylindole, and imaged on a Nikon A1 laser scanning confocal microscope (Nikon, Tokyo, Japan). Cerebellar white matter vacuoles were quantified using the NIS-Elements Annotate and Measure tool (Nikon). Thalamic vacuoles and GFAP immunoreactivity were quantified in Imaris 9.3 (Bitplane, Zurich, Switzerland) using 20× confocal z-stack images. Purkinje cell dendritic length and dendritic spine density were quantified in Imaris 9.3 using 60× confocal z-stack images. The Imaris Filament Tracing module was used to detect an automatic intensity threshold, subtract background noise, and generate dendrite computer reconstructions. Imaris parameters were set to detect dendritic spines between 0.2μm and 1.5μm in length. Purkinje cell dendritic total lengths were calculated by dividing total dendrite length/image by the number of Purkinje cell somas in the image. Images across treatment groups were analyzed with the same intensity thresholds. Statistical analyses were by 1-way or 2-way analysis of variance with post hoc Tukey test, or by 2-tailed Student t test. All animal experiments were conducted with University of California Davis Institutional Animal Care and Use Committee approval, and in accordance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Results

Cerebellar Nat8l mRNA abundance was similar in untreated 2-month-old CD and WT mice. Cerebellar Nat8l abundance was diminished in both CD and WT mice 2 weeks after intracisternal Nat8l gapmer administration, but had returned to pretreatment levels by 2 months post-Nat8l gapmer (Fig 1A). Nat8l gapmer administration initially lowered [NAA_B] in CD mice, but not in WT mice. [NAA_B] reduction persisted in some, but not all, CD mice 2 months post-Nat8l gapmer (see Fig 1B).

Accelerating rotarod retention times in untreated 2-month-old CD mice averaged less than half those in age-matched WT mice, but had increased significantly by 1 week post-Nat8l gapmer, and remained substantially above those in age-matched untreated CD mice 2 months post-Nat8l gapmer. The augmentative effect of Nat8l gapmer on CD mouse rotarod retention time was still present, but less marked, 3 months post-Nat8l gapmer (mean rotarod retention time 50.78 seconds in untreated CD mice vs 77.83 seconds in Nat8l gapmer-treated CD mice, $p = 0.0294$; n = 8 mice/group, 2-tailed *t* test). Intracisternal administration of Nat8l gapmer or control gapmer did not significantly alter accelerating rotarod retention times in WT mice, nor did intracisternal administration of control gapmer significantly alter accelerating rotarod retention times in CD mice (see Fig 1C).

PLP immunostaining indicated that cerebellar white matter vacuolar area was diminished in CD mice sacrificed 2 weeks postintracisternal Nat8l gapmer administration, and to a lesser extent in CD mice sacrificed 2 months post-Nat8l gapmer. $CC1^+$ oligodendroglial numbers in cerebellar white matter (not corrected for vacuolation) were similar in 2-month-old CD and WT mice, and in CD mice were not significantly altered by Nat8l gapmer administration (Fig 2A, B). Thalamic vacuole area was also diminished by Nat8l gapmer administration. Thalamic immunoreactive GFAP (not corrected for vacuolation) was substantially greater in 4-month-old CD than WT mice. Nat8l gapmer administration did not significantly diminish GFAP overexpression in CD mice (see Fig 2C, D).

Purkinje cell dendrites were shorter and had fewer synaptic spines per unit length in 4-month-old untreated CD mice than in 4-month-old untreated WT mice. By 2 weeks post-Nat8l gapmer administration to 2-month-old CD mice, Purkinje cell dendritic length and spines per unit dendritic length were not significantly different than those in untreated 4-month-old WT mice. The normalization of Purkinje cell dendritic length in the CD mice was still evident 2 months postintracisternal Nat8l gapmer (Fig 3).

Discussion

Though early postnatal AAV-mediated *Aspa* gene therapy prevents leukodystrophy in CD mice,[13,14] attempts to translate *Aspa* gene therapy to infants and children with symptomatic CD have thus far failed to reverse pre-existing neurological deficits or to prevent disease progression.[4] Neonatal intracerebroventricular administration of an AAV incorporating a short hairpin Nat8l inhibitory RNA to CD mice, which lowered [NAA_B] toward normal, also prevented development of leukodystrophy.[15] However, a sharp decrease in brain transduction by the AAV vector after the newborn period precluded evaluation of the potential of this therapy to reverse pre-existing ataxia, cerebellar vacuolation, and alterations in Purkinje cell morphology in the CD mice.

Intrathecal administration of a single-stranded LNA-ASO for modification of neuronal SMN2 splicing is now in clinical use in infants and children with spinal muscular atrophy,[16] and other chemically modified oligonucleotides have shown therapeutic promise in suppressing synthesis of toxic neuronal proteins in animal models of human neurodegenerative diseases.[17] The present study demonstrates that inhibiting [NAA_B] elevation by intracisternal administration of an LNA-ASO designed to suppress expression of the neuronal NAA synthesizing enzyme Nat8l[11] rapidly reverses ataxia, Purkinje cell dendritic atrophy, and cerebellar and thalamic vacuolation in young-adult CD mice. CD mouse cerebellar Nat8l mRNA abundance had returned to pretreatment levels by 2 months post-Nat8l gapmer, by which point [NAA_B] had already risen substantially, yet the marked improvement in accelerating rotarod performance of Nat8l gapmer-treated CD mice was fully maintained for 2 months, and was still substantially better than that in untreated CD mice 3 months post-Nat8l gapmer. Thus, substantial central nervous system tissue regeneration and functional improvement are feasible in young-adult CD mice. These results argue for consideration of intrathecal Nat8l gapmer therapy, either alone or as an adjunct to *Aspa* gene therapy for infants and children with CD. However, the downward drift in rotarod performance of the CD mice by 3 months post-Nat8l gapmer suggests that repeated intrathecal Nat8l gapmer administration would be required to achieve sustained therapeutic effect in CD.

How might elevated [NAA_B] elicit vacuolation and Purkinje cell dendropathy? Raising [NAA_B] in aspartoacylase-expressing mice, for example by engineering neuronal transgenic Nat8l overexpression, does not alter brain histology.[18] However, several human and murine mutations that perturb expression of astroglial ion channel–associated proteins cause vacuolar leukodystrophies.[19] Because astroglia express a sodium-coupled plasma membrane dicarboxylic acid transporter with high affinity for NAA, NaDC3 (encoded by *Slc13a3*),[20] vacuolation in aspartoacylase-deficient brains may be attributable to the osmotic effects of astroglial NAA overaccumulation,[3,5] and the rapid therapeutic response to Nat8l gapmer may be attributable to restoration of normal astroglial NAA content and osmolar homeostasis. It is unclear whether the Purkinje cell dendritic abnormalities in young-adult CD mice and the Purkinje cell losses previously reported in older CD mice[21] represent Purkinje cell-autonomous effects of elevated [NAA_B], or are secondary to astroglial or Bergmann cell dysfunction.

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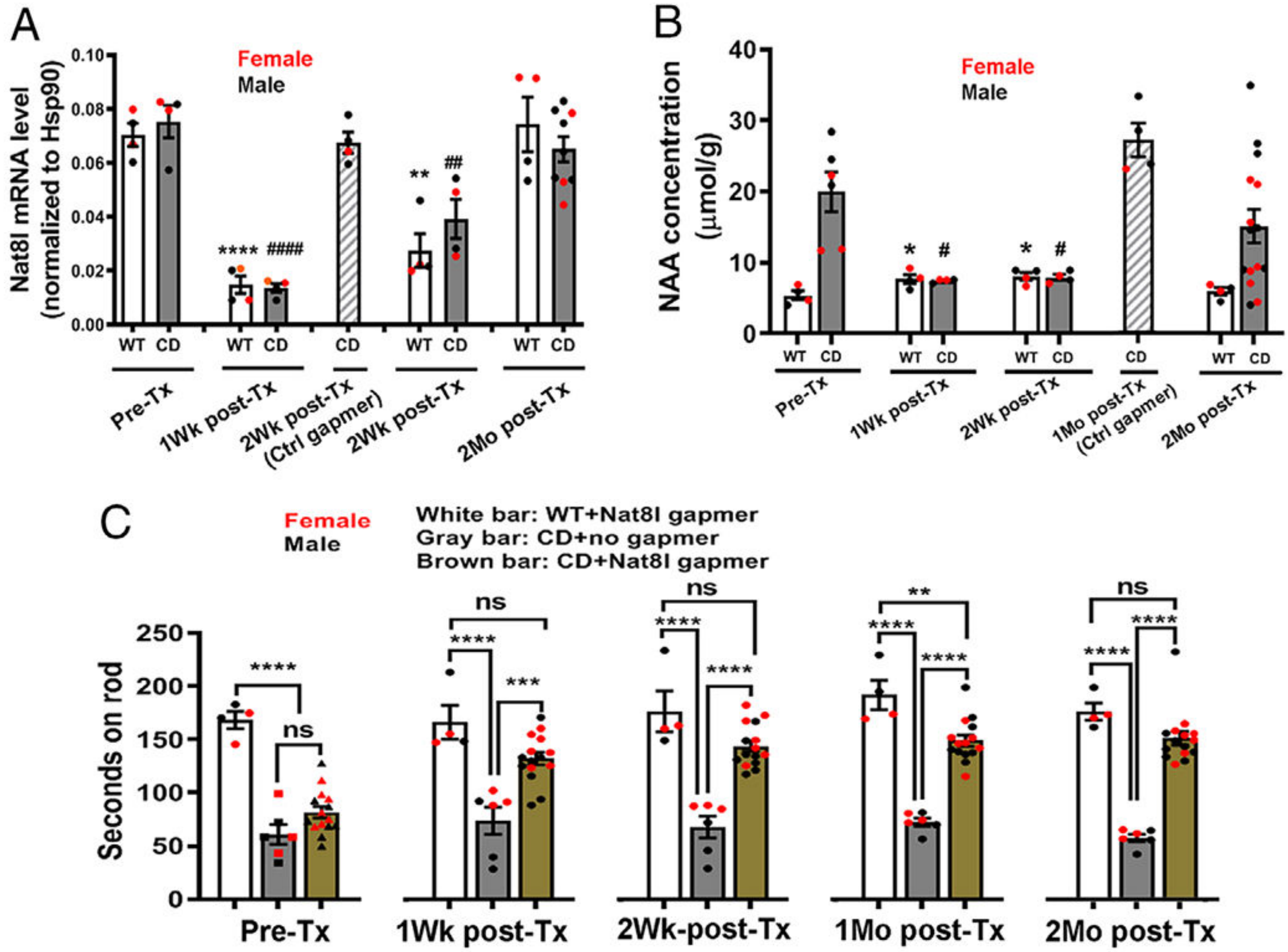

FIGURE 1:

Effects of intracisternal Nat8l gapmer on cerebellar Nat8l mRNA abundance ([NAA_B]) and accelerating rotarod performance. Each circle denotes data from an individual mouse (red female, black male). Mean and standard error of the mean (SEM) are indicated. (A) Quantitative real-time polymerase chain reaction for cerebellar Nat8l abundance. One-way analysis of variance (ANOVA), ####1 week posttreatment (post-Tx) Canavan disease (CD) different than pretreatment (Pre-Tx) CD, $p < 0.0001$; ****1-week post-Tx $Aspa^{WT/WT}$ control mice (WT) different than pre-Tx WT, $p < 0.001$; **, ##, and **2-week post-Tx WT and CD different than pre-Tx WT and CD, respectively, $p < 0.01$. Control gapmer did not significantly alter Nat8l mRNA abundance in CD mice (striped bar). (B) High-performance liquid chromatography assays for NAA (in micromole/gram cerebellar wet weight). Kruskal-Wallis test. * and #, 1- and 2-week post-Tx WT and CD different than pre-Tx WT and CD, respectively, $p < 0.05$. Control gapmer did not significantly alter [NAA_B] in CD mice (striped bar). (C) Accelerating rotarod retention times. ANOVA, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$. Accelerating rotarod performances of WT mice 2 weeks and 1 month postintracisternal Nat8l gapmer (168.5 ± 12.2, n = 6, 3 male [M]/3 female [F]; and 199.1 ± 12.8, n = 6, 3M/3F, respectively) did not differ significantly from those of untreated WT mice of the same age. Accelerating rotarod performances of CD and WT mice 1 month postcontrol gapmer were not significantly different from those of untreated age- and sex-matched CD and WT mice, respectively (WT no treatment 173.5 ± 8.3; treated WT 156.3 ± 6.3, mean ± SEM, n = 4 mice/group, $p = 0.1493$, 2-tailed *t* test). Weight gains (in grams) during the interval between 2 and 4 months of age were not significantly different between untreated WT (4.4 ± 0.7), untreated CD (4.1 ± 0.3), Nat8l gapmer-treated WT (5.5 ± 1.8), and Nat8l gapmer-treated CD (4.0 ± 0.8) mice ($p = 0.7815$, ANOVA). Two-way ANOVA at each time point indicated no significant differences between males and females in Nat8l mRNA, NAA concentration, or accelerating rotarod performance. ns = not significant.

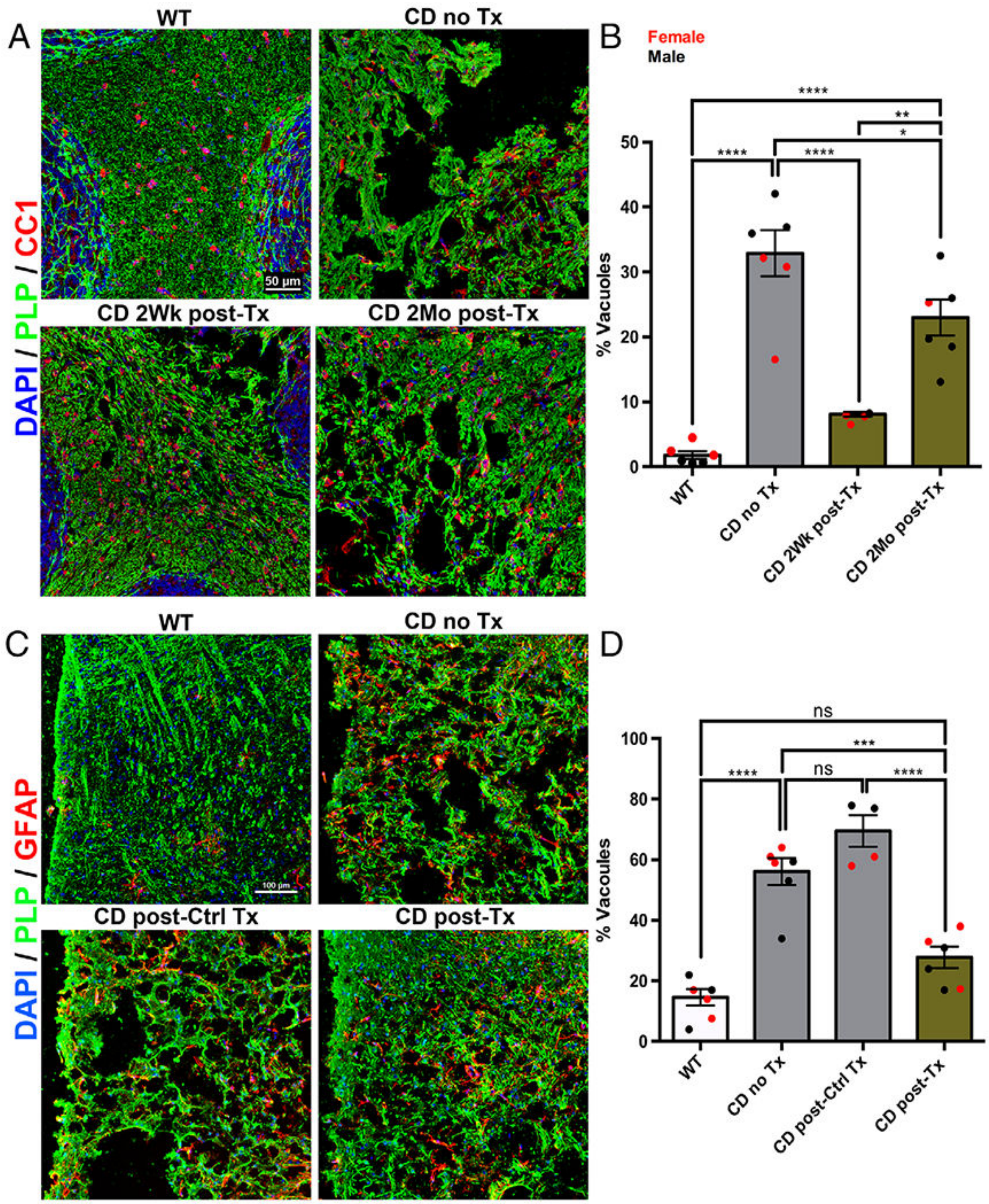

FIGURE 2:

Effects of intracisternal Nat8l gapmer on cerebellar white matter vacuolation and oligodendroglial numbers (A and B) and on thalamic vacuolation and GFAP immunoreactivity (C and D) in Canavan disease (CD) mice. (A) Images (z-stacks of four 2µm optical slices) immunostained for PLP and CC1, from representative $Aspa^{WT/WT}$ control mice (WT), CD without Nat8l gapmer treatment (CD noTx), CD 2 weeks after Nat8l gapmer treatment (CD 2Wk post-Tx), and CD 2 months after Nat8l gapmer treatment (CD 2Mo post-Tx) mice. Size bar = 50µm in each panel. (B) Cerebellar white matter vacuolar area. Each circle represents an individual mouse. Mean and standard error of the mean (SEM; n = 6 mice/group) are indicated. ****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$, analysis of variance (ANOVA). The effects of Nat8l gapmer treatment (Tx) on $CC1^+$ mature oligodendroglial numbers/square millimeter of cerebellar white matter (not corrected for white matter vacuolation) were: WT 330 ± 28, CD noTx 300 ± 36, CD 2Wk post-Tx 290 ± 12, and CD 2Mo post-Tx 340 ± 48 (mean ± SEM, n = 3/group; 1-way ANOVA not significantly different). (C) Images (z-stacks of four 2μm optical slices), immunostained for PLP and GFAP, from representative 4-month-old WT, CD noTx, CD 2 months post-Ctrl Tx (CD post-Ctrl Tx) and CD 2 months post-Nat8l gapmer treatment (CD post-Tx). Size bar = 100μm in each panel. (D) Thalamic vacuolar area. Each circle represents an individual mouse. Mean and SEM (n = 6 mice/group) are indicated. ****$p < 0.0001$, ***$p < 0.001$. Thalamic GFAP immunoreactivity (uncorrected for thalamic vacuole area and expressed as percent GFAP occupancy/field ± SEM) occupied 42.7 ± 7.9% in 4-month-old untreated CD mice, 2.92 ± 0.52% in 4-month-old wild-type mice, 33.3 ± 4.5% in 4-month-old control gapmer-treated mice, and 16.2 ± 3.0% in 4-month-old Nat8l gapmer-treated mice (n = 4–6 mice, sex matched/group). ANOVA with post hoc Tukey test indicated that GFAP immunoreactivity was more highly expressed in 4-month-old untreated CD than WT mice (73,457 ± 13,572 voxels vs 5,016 ± 898 voxels, mean ± SEM, n = 6, p <0.0001), and that there was no significant difference in GFAP immunoreactivity at age 4 months between CD mice that had received intracisternal Nat8l gapmer versus control gapmer at age 2 months (32,620 ± 2,708 vs 57,275 ± 7,667, mean ± SEM, n = 4, $p = 0.1851$). DAPI = 4′,6′-diamidino-2-phenylindole; ns = not significant.

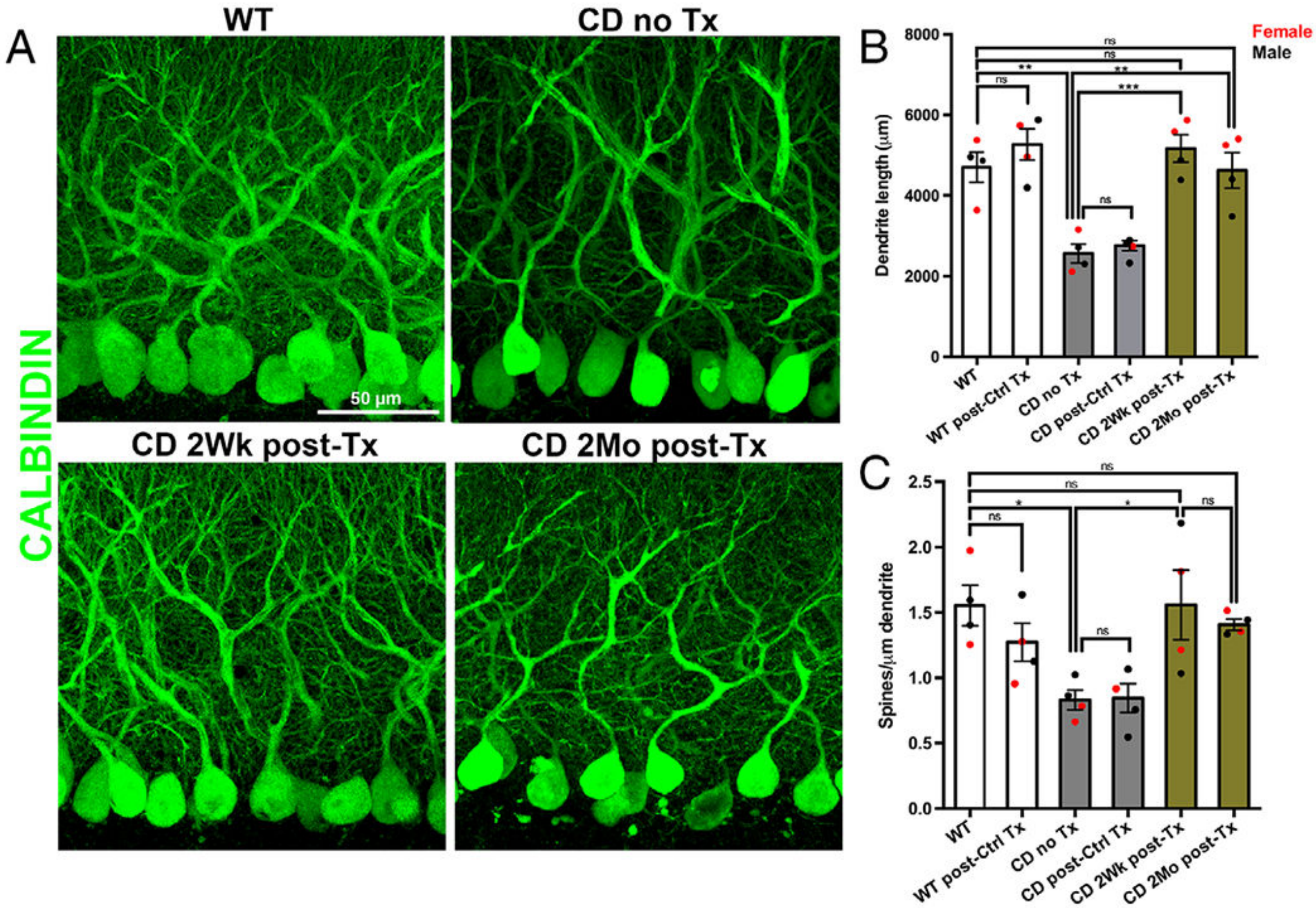

FIGURE 3:

Effects of intracisternal Nat8l gapmer on Purkinje cell dendrite length and dendritic spine density in Canavan disease (CD) mice. (A) Images (z-stacks of thirty 1 µm optical slices) from representative 4-month-old $Aspa^{WT/WT}$ control mice (WT), 4-month-old CD without treatment (CD noTx), CD 2 weeks following Nat8l gapmer treatment at age 2 months (CD 2Wk post-Tx), and CD 2 months after Nat8l gapmer treatment at age 2 months (CD 2Mo post-Tx) mice. Size-bar = 50µm in each panel. (B) Purkinje cell dendrite length. Each circle denotes data from an individual mouse. Mean and standard error of the mean (SEM; n = 4 mice/group) are indicated. ***$p < 0.001$, **$p < 0.01$; analysis of variance (ANOVA). (C) Purkinje cell dendritic spines/micron dendrite length. Each circle denotes data from an individual mouse. Mean and SEM (n = 6 mice/group) are indicated. *$p < 0.05$, ANOVA. Dendritic spines/micron dendrite length were not significantly more numerous in the CD 2Mo post-Nat8l gapmer Tx group than in either the CD noTx or CD 2Mo post-Ctrl Tx groups, CD 2Mo post-Nat8l gapmer Tx group. However, dendritic spines/micron dendrite length were significantly higher in the 2Mo post-Nat8l gapmer group than that in the combined 4-month-old CD noTx and CD 2 months post-Ctrl Tx groups ($p = 0.0354$).